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# PHYSIOLOGICAL ZOÖLOGY

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# THE INFLUENCE OF HIGH TEMPERATURE ON THE REPRODUCTIVE CAPACITY OF GUINEA-PIG SPERMATOZOA AS DETERMINED BY ARTIFICIAL INSEMINATION[1]

WILLIAM C. YOUNG
Hull Zoölogical Laboratory, University of Chicago

DURING a study of the histological changes in the guinea-pig testis following the application of temperatures slightly higher than that of the body, and, particularly, of the effects of such applications on the subsequent reproductive capacity, several problems arose which required additional attention.

In the earlier study (Young, 1927) water warmed to 46° C. was allowed to flow over the scrotum for 30 minutes. A progressive degeneration of the germinal epithelium set in immediately; pycnosis and chromolysis of the spermatocytes and younger spermatids being evident within 3 hours after the treatment. By the end of 12 days there had been a practical elimination of germ cells from the seminiferous tubules, except for a few of the strongly resistant spermatogonia. Spermatozoa contained in the epididymis were likewise strongly resistant and were found in some animals as long as 35 days after the treatment. Provided the treatment had not been too severe and spermatogonia survived, the latter served as the stem cells for a new germinal epithelium, and 45 days after the treatment many of the seminiferous tubules again appeared normal. Extension of the new epithelium throughout the tubules continued until about the

[1] This work was aided by a grant from the Committee on Sex Research of the National Research Council; grant administered by F. R. Lillie.

sixth month, by which time restoration had become complete except for a few atrophied tubules, destruction of which seemed permanent.

Beginning a day or two after the treatment and continuing for approximately 4 months, the injured males were allowed to mate with normal females at intervals of from 5 to 10 days. In this way breeding-histories were obtained for the period during which the histological changes were observed. Fertile matings continued from 7 to as many as 31 days after the treatment, their persistence being explained by the survival of spermatozoa contained in the epididymis at the time of treatment. A period of sterile matings then followed, which lasted usually until the seventy-fifth or one hundredth day. After this time litters again began to be produced, provided restoration of germinal epithelium had occurred; and a normal frequency of fertile matings was approached, if not attained.

Throughout the breeding histories the frequency with which abortions and stillbirths occurred seemed high, and a few definitely abnormal fetuses were found. The question arose, were these abnormal pregnancies attributable to the heat injury? They occurred in some histories following matings made during the first month after the treatment. In these cases they were thought possibly to be the direct result of a heat injury to spermatozoa already formed and in the epididymis at the time of treatment. In other breeding-histories, the abnormal pregnancies followed matings made 3 or 4 months after the treatment. In these cases they were thought possibly to be expressions of defects in the gametes caused by injury to the cells at an early stage in their history and transmitted to the spermatozoa during maturation.

It was relatively easy to test the effect of high temperature on the reproductive capacity of mature spermatozoa, thus disposing of this part of the problem. Furthermore, other investigators have demonstrated that injury to spermatozoa of the lower forms may lead to defective offspring, and I was particularly anxious, therefore, to study the effect of high temperature on the spermatozoa of the guinea pig as a representative mammal. Bardeen (1907) exposed toad sperm to X-rays prior to fertilization and obtained grossly abnormal larvae. O. Hertwig (1910) exposed frog sperm to radium emanations and obtained similar results. Dungay (1913) subjected

*Nereis* sperm to high temperatures and obtained abnormalities in the cleavage stages and in the trochophore larvae. G. Hertwig exposed frog sperm to X-rays and obtained eyeless embryos as well as embryos with defective eyes. Hinrichs (1926) exposed *Arbacia* sperm to ultra-violet radiations and obtained abnormal larvae.

The study was made by removing the inferior portion of the epididymis from the male guinea pig, subjecting it to varying temperatures, and inseminating normal females with the sperm during the oestrum. The results obtained are presented and discussed in the following pages.

I am indebted to Professor Frank R. Lillie for his assistance in making the study possible, and to Dr. Carl R. Moore for his interest and suggestions during the progress of the work.

## METHODS

The procedure of removing the tail of the epididymis from the organism, heating it at a given temperature, removing the sperm, and then injecting them into the uteri of females during the oestrum suggested itself as a more exact method of experimentation than that of merely allowing water, heated at a constant temperature, to run over the external surface of the scrotum. The former procedure enables the maintenance of a more careful control of the temperature than when the scrotal integument and tunica vaginalis separate the testis and epididymis from the heated water, the establishment of a more comprehensive range of exposures, and the employment of a large number of animals, thus minimizing the influence which an irregularity in one or two males might exert. The practicability of the method was demonstrated not only by the successful impregnation of the first few females into which sperm were introduced but also by the success attained by Iwanoff, Hammond and Asdell, Walton, and many others in other experiments.

When female guinea pigs were found in heat, the epididymides were removed from normal males, placed whole in a test tube containing about 8 cc. of Locke's solution, and heated in a water bath maintained at a constant temperature for a definite time. The epididymides were then macerated in 3–4 cc. of the Locke's solution in which they had been heated, and ½–1 cc. of the sperm suspension

injected through the vagina into the body of the uterus by means of a catheter made to fit into this part of the genital tract or even an ordinary medicine dropper. The female was then held as quietly as possible, ventral side up, for no less than 4–5 minutes, a small cotton plug was inserted in the vagina at the end of this time, and she was placed in an isolation pen. The glassware which was used was clean but not sterile.

One hundred and sixty females were inseminated in this manner with sperm which had been subjected for 30 minutes to one of the following temperatures: 38°, 40°, 42°, 44°, 45°, and 46° C.—a range which extends from guinea-pig body temperature, 39°, to the highest temperature guinea-pig sperm will survive following an exposure of 30 minutes, namely, 46°. The exposures at 38° and 40° are so near the normal body temperature that it seemed permissible to regard inseminations made with these sperm as controls. It was usually possible to obtain from two to six females for insemination simultaneously, and this was done (1) with the hope that a possible effect on one litter might also be found on a second litter and (2) in the interest of economy.

## EXPERIMENTAL AND DISCUSSION

A tabular review of the experiment is given in Table I. It will be observed that there does not seem to be much effect on the fertilizing capacity until an exposure of 45° for 30 minutes is reached, the

TABLE I

| Temperature Exposure for 30-Minute Period | No. of Females Inseminated | Pregnancies | | Failures of Fertilization | No. of Abortions and Stillbirths | Percentage of Abortions and Stillbirths to Pregnancies |
|---|---|---|---|---|---|---|
| | | No. | Percentage | | | |
| 38° C..... | 26 | 16 | 61.5 | 10 | 5 | 31.2 |
| 40° C..... | 28 | 18 | 64.3 | 10 | 2 | 11.1 |
| 42° C..... | 31 | 16 | 51.6 | 15 | 5 | 31.2 |
| 44° C..... | 25 | 13 | 52.0 | 12 | 2 | 15.4 |
| 45° C..... | 25 | 7 | 28.0 | 18 | 2 | 28.6 |
| 46° C..... | 25 | 2 | 8.0 | 23 | 0 | ∞.0 |

percentage of impregnations falling off sharply at this point. Following an exposure of 46° for 30 minutes, impregnation is exceptional; and following an exposure of 47° for 30 minutes (not shown in the table), spermatozoa show no more than a slight motility, if any.

Most interesting is the fact revealed above that the frequency of abortions and stillbirths was no greater following inseminations with sperm heated at the higher temperatures than it was following inseminations with those heated at body temperature and regarded, therefore, as controls. As the table shows, inseminations with sperm exposed to temperatures approximately equivalent to body temperature were followed by as high a frequency of abortions and stillbirths as were inseminations with sperm which had been exposed to the highest temperatures at which fertilizing capacity is retained. Sperm which had been subjected to these higher exposures were injected into fifty females and, although a marked loss of fertilizing capacity resulted, there is no evidence of an injury which manifests itself by some defect in the offspring.

The additional fact should be emphasized that there was no concentration of abnormal pregnancies among females into which sperm from the same batch had been injected. One or more normal litters may have been produced, single cases of abortion may have occurred, and there may have been sterile inseminations among such a group of females; but in no instance did two or more abnormal pregnancies follow inseminations with sperm heated at the same time.

It would seem, therefore, to be impossible to attribute the abortions and stillbirths which occurred in this and the earlier experiment to the heat injury of spermatozoa contained in the epididymis at the time of treatment. If the reproductive capacity of male germ cells can be demonstrated to be altered, in any way except by being destroyed completely, following exposure to high temperature, it seems, at present, that experiments will have to be confined to the immature stages and not to spermatozoa which have passed into the epididymis. It may be that an injury to spermatids, spermatocytes, or spermatogonia might not prevent a continuation of the maturation process and that defective sperm would result. A study in which an attempt is being made to administer such a sublethal injury to the immature cells has been started and the results will be reported later.

The ease with which female guinea pigs can be successfully impregnated artificially, as demonstrated in this study, suggests a re-

emphasis of the method as a means of investigating further the physiology of mammalian spermatozoa. Despite the extensive work done in agricultural research laboratories by Iwanoff (1903, 1907, 1913, and 1922), Hammond and Asdell (1926), and Walton (1927), and some clinical studies, it still appears that many fail to recognize the possibilities of experimentation by means of artificial insemination of mammals. The method permits the same breadth of procedure and precision in studies of mammalian sperm that are possible in studies of spermatozoa of the lower forms, but which cannot be extended to mammalian studies as long as the sperm remain in the organism. In addition, experimental results can be obtained in terms of reproductive capacity rather than merely in terms of changes in vitality as recorded by such standards as speed and duration of motion—certainly a desirable objective in investigations of mammalian sperm.

The capacity of epididymal sperm for fertilization which is followed by normal development and parturition without the addition of secretion from the other accessory sex glands, such as the prostate and seminal vesicles, again was demonstrated and should receive brief re-emphasis. The activating influence attributed to prostatic secretion by Hirokawa (1909) apparently is provided by the salts in Locke's solution. The vaginal plug, a product of the secretion of the seminal vesicles, can be dispensed with entirely if the animals are handled carefully or if its place is taken by a small wad of cotton or some improvised substance.

## CONCLUSIONS

1. Spermatozoa contained in the epididymis can be exposed to temperatures ranging from 38° C. to 46° C. for 30 minutes and then used for artificial insemination without a marked loss of fertilizing capacity until a temperature of 45° is reached. The percentage of impregnations then falls off abruptly, and at 47° even motility is lost.

2. Offspring from females inseminated with sperm exposed to the higher temperatures, 45° and 46°, are fully as vigorous as those from females inseminated with sperm exposed to body temperature for the same length of time.

3. The frequency of abortions and stillbirths was as great among

females inseminated with sperm exposed to the lower temperatures as it was among females inseminated with sperm exposed to the higher temperatures.

4. It would seem, therefore, that if the reproductive capacity of male germ cells is altered by exposure to high temperature, the effect must be produced on cells in the immature stages and not on spermatozoa contained in the epididymis.

5. Artificial inseminations have again been demonstrated to be a practical means of studying the physiology of mammalian spermatozoa.

6. The capacity of epididymal sperm for fertilization without the intervention of products from the accessory sex glands such as the prostrate and seminal vesicles has again been demonstrated.

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# NEW EVIDENCE FOR THE FUNCTION OF THE SCROTUM[1]

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## I. INTRODUCTION

THE results of studies made in this laboratory by Moore and his collaborators have shown conclusively that the scrotum is a local thermo-regulator, functioning to reduce the temperature environment of the testes below that of the general body temperature (Moore, 1924*a*, 1924*b*, 1924*c*, 1926*a;* Moore and Quick, 1924*a*). In brief, it was found that a normally functioning testis of the adult guinea pig will lose practically all its germinal epithelium when the testis is elevated from the scrotum into the abdomen; the seminiferous tubules show marked degeneration within a week after confinement of the testis in the abdomen, and within 2–3 weeks the tubules are reduced to shrunken, vacuolated, and empty canals except for a single basal row of nuclei (presumed to be Sertoli elements). All spermatogenesis ceases, and the testis shows no spermatogenetic activity as long as it remains in the abdomen. If, however, it is replaced in the scrotum after degeneration of the germinal epithelium has occurred, spermatogenesis is re-established and spermatozoa are again produced.

The various lines of evidence, including measurements of temperature (Moore and Quick, 1924*a*), experimental cryptorchidism (Moore, 1924*b*, 1926*a*, 1926*b*), applications of higher temperatures to the scrotum (Fukui, 1923; Moore and Chase, 1923; Moore, 1924*c;* Young, 1927), as well as the studies on vasectomy (Moore and Oslund, 1924; Oslund, 1924; Moore and Quick, 1924*b*), have demonstrated that the effective agent is the higher temperature of the abdomen. It was shown that in the guinea pig the temperature of the abdomen was 2°–3° C. higher than scrotal temperature. Hammond and Asdell (1926) have reported comparable differences between abdominal and scrotal temperatures in the rabbit. The func-

[1] This investigation has been aided by a grant from the Committee for Research in Problems of Sex of the National Research Council; grant administered by Professor F. R. Lillie.

tion of the scrotum is therefore revealed to be that of a temperature-regulator for the testes.

Despite the decisiveness with which the regulatory property of the scrotum has been demonstrated as it influences germ-cell production in the testis, it was not so clearly indicated that the temperature regulatory properties of the scrotum extended in a similar manner to the spermatozoa once they were differentiated and stored within the epididymides. A logical supposition might be that such an influence did not extend to the spermatozoa themselves, since, on mating, the spermatozoa are introduced into the vagina, uterus, and oviducts where the temperature is essentially the same as that of the peritoneal cavity. Many authors believe that motile sperm exist in the female genital passages for long periods of time, especially in the human, thus implying that such temperatures have no effect on these cells.

A study of the effect of abdominal temperature on fully developed spermatozoa was begun (Moore, 1928) but was discontinued before completion. The problem was suggested to me for further study by Dr. Carl R. Moore, and my observations are reported in the following pages. They clearly reveal that the heat regulatory function of the scrotum does extend to the differentiated spermatozoa stored within the epididymides.

I take great pleasure in acknowledging my indebtedness to Dr. Moore for the suggestions and for guidance and discussions during the pursuit of this study.

## II. MATERIAL AND METHOD

For this study young adult guinea pigs and white rats have been used as the experimental animals. The method employed is that introduced and more fully described by Moore (1928). Operating through a low mid-abdominal incision, one or both testes were carefully removed from the epididymis, and the gonad proper removed from the animal. The epididymis with its content of millions of spermatozoa, still retaining its normal ductus deferens and blood and nerve connections, was confined in the abdomen by closing the inguinal canals. In this manner the epididymis was confined to the abdomen, and its contained spermatozoa served at later periods as indicators of effectiveness. At properly timed intervals, the epididy-

mis was removed from the abdomen, finely hashed with scissors in a small quantity of physiological saline solution, and studied at once under the microscope for spermatozoan motility.

Two types of operations were practiced on both the rat and the guinea pig. In the first type, one testis was removed from the animal, the corresponding epididymis being confined in the abdomen and serving as the test epididymis; while the opposite testis remained normal. In the second type, both testes were removed, and each epididymis was confined in the abdomen. The two types differ fundamentally in that the first series of observations was made on animals possessing one normal testis, and therefore, supplied with the internal secretions from this organ. The second series of observations was made upon animals from which the testis hormone had been excluded by testis removal. For controls on spermatozoön motility from epididymides remaining in the scrotum, I have depended upon the data supplied from animals reported by Moore (1928), a number of which were checked by me at the time of observation.

In recording observations of spermatozoön motility, I have chosen to represent movement obtained from a normal epididymis as **** and the entire absence of motility by o. The weakest movement on the part of a few spermatozoa would thus be designated*; ** and *** would indicate still stronger spermatozoön motility, but yet not as strong as normal. The observations here reported are based upon an examination of fifty operated animals.

### III. OBSERVATIONS ON BILATERAL ISOLATIONS OF THE EPIDIDYMIDES CONFINED IN THE ABDOMEN

In an attempt to determine what effect, if any, the higher temperature of the abdomen has upon the life of the spermatozoön, as indicated by its capacity for motility, both testes of the animal operated were removed and the epididymides elevated into the abdomen. In order to be able to appreciate the meaning of my observations on sperm motility after such operations, it must be recalled that Moore (1928) determined that spermatozoa of the guinea pig retained their capacity for motility for a period of 23 days in an epididymis which was isolated in the scrotum when both testes had

been removed. This was likewise noted by Benoit (1926). The corresponding period for the rat was found to be 18 days.

A. *Guinea pig.*—Table I gives the results of my observations on the motility of guinea-pig spermatozoa recovered from epididymides that had been confined in the abdomen for different periods of time. This table reveals that spermatozoa recovered from epididymides

TABLE I

BILATERAL ISOLATION AND CONFINEMENT OF EPIDIDYMIDES IN THE ABDOMEN. (GUINEA PIG)

| No. of Days after Operation | Motility | No. of Days after Operation | Motility |
|---|---|---|---|
| 9 | ** | 13 | o |
| 10 | ** | 13 | o |
| 10 | * | 13 | o |
| 11 | * | 13 | o |
| 11 | o | 13 | * |
| 11 | o | 14 | o |
| 11 | o | 14 | o |
| 12 | * | 14 | o |

TABLE II

BILATERAL ISOLATION AND CONFINEMENT OF EPIDIDYMIDES IN ABDOMEN. (RAT)

| No. of Days after Operation | Motility | No. of Days after Operation | Motility |
|---|---|---|---|
| 5 | * | 7 | * |
| 5 | * | 7 | o |
| 6 | * | 7 | o |
| 6 | o | 7 | o |
| 6 | o | 8 | o |
| 7 | * | | |

of the guinea pig that had been confined in the abdomen for a period of approximately 12 days have lost their power to be activated by saline solutions. Nine days' confinement results in the loss of the capacity for motility on the part of a large percentage of the sperm, and a reduction of this power in all of the others. One case of 11 days' confinement, one of 12, and one of 13 days were noted in which the slightest movement of a few spermatozoa could be detected. Against this number, ten other cases of 11–14 days' confinement failed to show any sperm exhibiting movement. It thus becomes clear that confinement in the abdomen reduces the length of life of

these cells, as indicated by their capacity for motility, from 23 days (when located in the scrotum) to approximately 12 days.

B. *The rat.*—Observations duplicating the foregoing have been made on the rat, and these data are presented in Table II. The normal expectation for retention of the capacity for motility of rat spermatozoa from isolated scrotal epididymides is 18 days (Moore, 1928). Table II shows that when the epididymides are confined within the abdomen this period is reduced from an expected 18 days to 6 or 7 days.

In the case of the rat, therefore, as well as in that of the guinea pig, confinement of the spermatozoa within the abdomen very materially shortens the length of time their capacity for motility is preserved.

## IV. OBSERVATIONS ON UNILATERAL ISOLATIONS OF EPIDIDYMIDES CONFINED IN THE ABDOMEN

In the previous section we were dealing with animals in which both testes were removed. As Moore (1928) has shown that the hormone produced by the testis lengthens the capacity for motility of guinea-pig spermatozoa from 23 to 70 days in epididymides isolated in the scrotum, it was of importance to find out whether the hormone would affect the spermatozoa in abdominally isolated epididymides in a similar manner. Accordingly, one epididymis was elevated into the abdomen (the corresponding testis having been removed), while the opposite testis was left normal to supply the hormone.

TABLE III

UNILATERAL ISOLATION OF EPIDIDYMIS IN THE ABDOMEN. (GUINEA PIG)

| No. of Days after Operation | Motility | No. of Days after Operation | Motility |
|---|---|---|---|
| 6 | *** | 15 | o |
| 10 | *** | 15 | o |
| 11 | ** | 15 | o |
| 13 | o | 15 | o |
| 14 | * | 16 | o |
| 15 | o | | |

A. *Guinea pig.*—Table III presents my observations made on guinea pigs following operations as described above. It can be seen that the results obtained from this group agree with the findings

presented in Table I. The spermatozoa have retained their capacity to show motility for only 14 days after operation, despite the fact that one testis was present and carrying on its normal spermatogenetic activity. Despite the fact that the internal secretion of the testis lengthens the life of spermatozoa when these are located in the scrotum, it does not do so when the sperm are subjected to the influences of abdominal environment.

B. *Rat.*—Duplications of the foregoing experiment have been made on the rat, and the results are given in Table IV. The effect of the abdominal environment is again shown by these observations. When one testis is removed from the rat and the corresponding epidid-

TABLE IV

UNILATERAL ISOLATIONS AND CONFINEMENT IN THE ABDOMEN. (RAT)

| No. of Days after Operation | Motility | No. of Days after Operation | Motility |
|---|---|---|---|
| 4 | ** | 6 | o |
| 5 | ** | 6 | o |
| 5 | ** | 6 | o |
| 5 | * | 6 | o |
| 6 | * | 6 | o |
| 6 | o | 7 | o |

ymis allowed to remain in the scrotum, the spermatozoa contained within it will retain their capacity for motility up to 30 days (Moore, 1928; and personal observations). When the same operation is performed but the isolated epididymis is confined in the abdomen, this period is reduced from 30 days to 6 days. Furthermore, when one compares the results shown in Table IV with those of Table II, it will be seen that, as in the guinea pig, the presence of one testis supplying its internal secretion does not affect the period of life of the spermatozoön when it is confined within the abdomen.

## V. DISCUSSION

The observations presented above on the differential effects of the abdominal environment and the scrotal environment on the persistence of the life of the spermatozoön are of especial importance for the bearing they have on the problem of scrotal function.

The spermatozoa of the guinea pig have, we may say, a limited period of existence. It is apparent that after removal of both testes

the spermatozoa present in the epididymides gradually perish; the length of time they will persist and show motility when suspended in saline solution, when the epididymis remains in the scrotal position, is approximately 23 days. Their vitality gradually wanes after their formation, until their death. This fact can be appreciated by observing spermatozoa suspended in physiological saline solution taken from epididymides on the fifth, tenth, fifteenth, and twentieth day after testis removal. In the case of the shorter periods of isolation, the vast majority of spermatozoa will be seen to have vigorous motility immediately after suspension in the saline solutions; and gradually increasing length of time of observations after isolation shows not only that fewer sperm are in motion but that the motion of the entire lot is progressively weaker as the isolation time is extended. After the twentieth day, a very careful observation must be made to discern any movement among the thousands or millions of spermatozoa in the suspension. Perhaps only 1 per cent or a fraction of 1 per cent, may exhibit a weak vibratile contraction with little or no translation. Movement of this type has been discerned up to 23 days after the testis had been removed.

The life-span of the spermatozoa in the isolated epididymis can be greatly increased, however, if a testis remains in the animal and produces its internal secretion. It matters not whether such a testis be producing germ cells, for Moore has shown that experimental cryptorchid testes, degenerate for longer than a month, will have a stimulating influence on the spermatozoa in the opposite epididymis. He has utilized this increase of life of the spermatozoön as an indicator for the hormone of the testis (1927, 1928). Under the influence of a supply of testicular internal secretion the spermatozoa will live and still have the capacity to exhibit motility for a period of 60–70 days. These periods of time apply to the spermatozoa of the guinea pig when the isolated epididymis is in the scrotum. For the rat, comparable periods of time are approximately 18 days without a supply of testis hormone and 30 days when the hormone is present.

When, however, the isolated epididymis of the guinea pig is elevated into the abdomen, both testes having been removed, the period over which the spermatozoa exhibit capacities for movement is reduced from an expected 23 days to an actual 12–13 days. A

more striking difference appears when one epididymis is isolated (with corresponding testis removal) and the opposite testis supplies the hormone. If the epididymis is located in the scrotal position, spermatozoa would be expected to remain alive for 60 days or longer; but when it is located in the abdomen, the period is reduced to approximately the same period as before, namely, to 14 days. For the rat, similar findings appear. In no case have I found spermatozoa to show movement when the epididymis has been confined in the abdomen for longer than 7 days. Had the isolated epididymis been in the scrotum instead of in the abdomen, spermatozoa would have remained alive for 30 days.

Since the earlier observations in this laboratory have so conclusively shown that the higher temperatures of the abdomen were responsible for the testicular degeneration, and the scrotal influence has been recognized as the controlling factor, the data presented in this paper supply further evidence of the scrotal efficiency in maintaining the life of the spermatozoa after these have been completely formed.

It can be seen that the abdominal environment is effective so quickly that the life of the spermatozoön is not extended at all by the hormone of the testis.

## VI. SUMMARY AND CONCLUSIONS

1. Spermatozoa in an epididymis of the guinea pig from which the testis has been removed will live and become activated by suspension in physiological saline solution for a period of 23 days, provided the epididymis remains in the scrotum; when the epididymis is confined in the abdomen, the sperm show a capacity for motility for only 13–14 days.

2. After similar operations in the rat, the spermatozoa will show motility for 18 days when the epididymis is in the scrotum, but for only 5 days when it is in the abdomen.

3. Supplying the testis hormone, though previously shown to increase the life of spermatozoa by two to three times (Benoit, Moore), when the epididymis is located in the scrotum has no effect in prolonging the life of the spermatozoa when the epididymis is confined in the abdomen.

4. The difference in viability of the spermatozoa when confined in an epididymis in the scrotum and in one in the abdomen shows that the thermo-regulatory function of the scrotum extends to the formed spermatozoa as well as to the germinal epithelium of the testis.

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# AN EXPERIMENTAL STUDY OF FISSION AND RECONSTITUTION IN STENOSTOMUM

(Forty-six figures)

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## INTRODUCTION

THIS paper presents comparative data of the processes of fission and reconstitution in three closely related species of flatworms and attempts a physiological interpretation of the facts observed. The relation of the dominant apical end of an organism to the reconstitutive capacity of various levels of the body has been extensively investigated in many of the simpler organisms, in all of which the apical end normally controls more or less completely the differentiation and organization of the rest of the body. Evidence from various sources indicates that this control consists primarily of a transmissive process of some kind which undergoes a decrement basipetally. In the flatworm, *Planaria*, a sudden posterior rise of the gradient marks the location of a secondary dominant center of a new individual in long worms. This new zooid, and others that may arise posterior to it, are recognizable only physiologically, while they remain attached to the anterior zooid. But in the rhabdocoel turbellarian *Stenostomum*, physiological isolation of a posterior region results in the morphological development of the new individual before fission occurs, so that the worm normally exists as a chain of zooids in various stages of development. Moreover, instead of the zooids being linearly arranged as in *Planaria*, in the *Stenostomum* chain the order as regards time of origin is more complex, e.g., the first zooid, having divided once, may divide again to give rise to a third zooid, long before the second has separated. Similarly the second may divide once or more while attached to the anterior zooid. By continued fission of attached zooids, chains of as many as eleven zooids may be formed; usually, however, separation occurs before this length is attained, so that chains of three, four, and five zooids are the most common forms found in nature.

Such an arrangement of head regions raises the question as to the influence of these possibly conflicting dominant regions on reconstitution: What, for example, will be the result of experimental section between two fission planes of the chain? The accounts of previous investigators of the problem of reconstitution in *Stenostomum* show considerable disagreement, which may be due to confusion in identification of species. Ritter and Congdon (1900) described the result of experimental section of a dividing *S. leucops*, in another plane than that of normal fission, as an inhibition of the normal plane next posterior to the plane of section and a migration of the structures developing at that fission plane to the plane of section. This result was thought to be brought about by a migration of the ganglionic masses normally found at a fission plane to a similar position at the anterior cut surface where a new head developed, the former posterior fission plane disappearing entirely in the course of this migration.

Child (1903) reported that the reconstitution of both *S. grande* and *S. leucops* was marked by the occurrence of reduction and resorption of anterior partial zooids and younger zooids by older posterior zooids in the absence of the head of the chain. When the chain was cut at various levels between fission planes, the results depended on the degree of development of the fission planes in the cut piece. If a piece containing a very young fission plane was cut from a chain, the fission plane disappeared and a single perfect individual was formed from the parts which originally belonged to two different zooids, and a head was reconstituted at the anterior cut surface. If the included fission plane was more fully developed, it remained and the part anterior to it was completely resorbed by the part posterior to it, the head of the resulting new individual being the head which was forming at the fission plane. The relative size of the parts anterior and posterior to the fission plane did not affect the result unless the posterior piece were very small. It was always the anterior part of the cut piece which was resorbed, never the posterior; while the actual resorbing zooid was always the oldest or most advanced in development of the complete or partial zooids of the cut piece. If the fission plane was still further advanced in development, the portion anterior to it was only partly resorbed;

it reconstituted a head and became a new zooid, though at first it decreased in size owing to partial resorption. Child found no evidence that migration of ganglia to the anterior end of the cut piece ever occurred, and pointed out that the facts precluded such a possibility.

Hartmann (1922) has recorded results of sectioning *S. leucops*, incidental to work on another problem, in which he stated that both anterior and posterior halves of a single zooid worm reconstitute new heads after section. Remarks on the form used indicate that it does not correspond with the species called *S. leucops* in this paper. Ruhl (1927) published a short paper which described the reconstitution of *S. leucops* and of *S. langi*, a European form never reported in this country but interesting because of the similarity of its reconstitutional behavior to that of *S. leucops* described in this paper. His account of the reconstitution of cut pieces of one-zooid *S. leucops* confirmed Hartmann's observations and extended them to show that nothing less than one-quarter of the entire body length reconstituted a head. For pieces with a fission plane, his results differed from those of Child, and Ritter and Congdon, in that he found only the smallest fraction of the anterior zooid to be resorbed under any circumstances. With more anterior cuts, the posterior zooid separated and the anterior piece either reconstituted a head or disintegrated. Similar pieces of *S. langi* divided at the fission plane without resorption; if the anterior partial zooid was long, new fission planes appeared in it and the resulting zooids also separated. The anterior piece then reconstituted a head, apparently in all cases. Ruhl contrasted this formation of new fission planes in partial zooids with the situation in his *S. leucops*, in which a new fission plane did not appear after head removal until a new head was reconstituted.

This problem was suggested by Professor C. M. Child, to whom I am deeply indebted for advice and criticisms; my thanks are also due to Dr. L. H. Hyman for suggestions during the course of the work.

## DESCRIPTION OF SPECIES

The fact that the present account of the process of reconstitution in *S. leucops* differs from those in the literature, which in turn show considerable variation, suggests that the discrepancies result from confusion in the identification of species or, perhaps, from the

existence of several varieties of *S. leucops* which differ in their reconstitutional behavior. The three species used in the present study were identified according to von Graff (1911, 1913) as the following:

*Stenostomum grande* Child
*Stenostomum tenuicauda* v. Graff
*Stenostomum leucops* Dugès(?)

Of the first two species, there is no doubt as regards the correct identification, since both agree with von Graff's descriptions.

The species tentatively identified as *S. leucops* in this paper corresponds to the description of that species given by von Graff save for one important diagnostic feature, the character of the patelliform organs. Instead of being dish-shaped and composed of many globular bodies as in the other two species, they were found to be hollow or vesicular, consisting of a roughly heart-shaped, highly refractive vesicle, attached to the anterior wall of which could barely be seen with an oil-immersion lens an opaque inclusion. Associated with this glistening vesicle was a larger translucent sphere, which was anterior to the former when the worm was quiet. In every other way this form appeared to be the *S. leucops* described not only by von Graff but in an extended microscopic study by Ott (1892). This form has no tail such as the other two forms have; the posterior end of the zooid decreases to about half its former width, but the endoderm extends to the posterior tip of this narrowed portion. The form of the head and pharynx differs distinctly from that of the other two species: In *S. leucops* the tip of the head forms an acute angle, that of both other species is broad and shovel-shaped. The pharyngeal region is relatively longer in *S. leucops;* the pharynx itself is free from conspicuous folds, as in *S. grande,* and from numerous large gland cells, as in both *S. grande* and *S. tenuicauda.*

The form called *S. leucops* in this paper resembles the European species *S. langi* in method of head-reconstitution, as described by Ruhl (1927), but differs morphologically both from that species and from *S. agile.*

## METHODS AND PROCEDURE

Both freshly collected worms and those cultured in the laboratory were tested for reconstitutional capacity. The former were ob-

tained by bringing masses of such aquatic plants as *Elodea*, *Chara*, and *Myriophyllum* into the laboratory and packing them in glass jars with only enough water to cover them. Within 24 hours the worms, when present, began to appear at the surface of the water or on the sides of the container near the top, whence they could be easily removed to fresh water by means of a finely drawn pipette. Worms so obtained were always sectioned within 48 hours of the time of collection. In order to provide a constant and plentiful supply of material, various culture media for the worms were tried. The standard adopted as being most reliable and convenient was a protozoan wheat culture, made by adding 20 grains of previously boiled wheat to a liter of treated well water. Ordinary well water was found to precipitate carbonates on standing which interfered with clear vision in the watch glasses. Treated well water was prepared from this by boiling it vigorously for about 10 minutes, filtering, and aërating; it differs from ordinary well water only in the amount of Ca present, which has been reduced from 49.2 parts per million to 26.1. After 3 days the culture was seeded with *Paramecium* and a few of the worms to be cultured were added, which multiplied rapidly as the protozoan content increased. Such stock cultures ran for about 2 months without the addition of more wheat; they were kept at room temperature unless otherwise stated. New cultures were successively started and used in turn as the source of worms. All three species multiplied readily under such conditions, especially *S. leucops*.

All the work on reconstitution was done under the binocular dissecting microscope because of the small size of the worms, the mean length of *S. leucops* with three zooids being just over 1 mm. The procedure in cutting a piece for the determination of its reconstitutive capacity was as follows: A worm which appeared to be of the desired size or number of zooids was picked from the culture by means of a fine pipette and placed in a drop of water under the microscope. If found satisfactory, enough of the water was withdrawn to force the worm to glide about on the glass rather than swim freely. For making the cuts across the body, a Knapp's knife-needle was used. The cut piece to be observed was then placed in a dry Syracuse watch glass which was filled with water after it was certain the

piece was actually transferred. Unless otherwise stated, this water in which the piece lived after section was the treated well water. Each cut piece was individually isolated.

Since Child had described the reconstitutional process in *S. grande* as being completed within 72 hours after section, and hence requiring frequent observation to follow the course of reconstitution, observations at intervals of several hours were made on similar pieces of the *S. leucops* of this paper. The failure to find evidence of resorption in the reconstitution of this species led to the practice of taking observations daily or every other day. None of the regulatory processes in *S. leucops* proceed at such a rate that they cannot be recorded and interpreted by these means. In recording the results the first clear indication of the appearance of ciliated pits in their proper position at the anterior end marked the piece as being capable of reconstitution. The ganglionic masses whose formation precedes that of the ciliated pits are distinguishable only with difficulty at this stage and cannot be used as indicators of the first signs of reconstitution. Once the ciliated pits form, it is only a matter of some 24 hours before the mouth, pharynx, and normal head form appear. No reconstitution of any kind beyond closure of the wound takes place at the cut anterior surface of the piece unless the ganglia and their associated external indications, the ciliated pits, form. In ordinary procedure the first observation indicating the presence of ciliated pits was recorded as the time at which the piece reconstituted. Unless they did appear, the piece was recorded as headless.

### RECONSTITUTION OF S. GRANDE

The study of the process of reconstitution in *S. grande* confirmed fully the very complete description of the process given by Child (1903) for this species. It was indicated in the preceding summary of this work that pieces of *S. grande* may reconstitute a head in one of two ways, depending on the presence and development of the fission plane or planes in the cut piece: either a new head is reconstituted at the anterior end without resorption in pieces containing no fission plane, or the presence of fission planes in the cut piece results in reconstitution by resorption of the anterior partial zooid and younger complete zooids by the most advanced head region of

the posterior fission planes. For comparison with the other species, sets of cut pieces of both freshly collected and laboratory-cultured worms were kept under observation to determine their head frequency and the time required for head formation. Table I gives the head frequency, or percentage of cut pieces reconstituting heads after excluding those pieces which died within 24 hours after the operation, of worms from the two sources. These pieces consist of headless tail zooids, isolated from worms of two to five zooids, by section posterior to the head of the zooid; none of them contained a visible fission plane at the time of operation, and no fission planes appeared in the cut pieces during reconstitution. The head was re-

TABLE I

RECONSTITUTION OF FRESHLY COLLECTED AND LABORATORY-GROWN S. GRANDE

| | Freshly Collected Material | Cultured for 3 Months |
|---|---|---|
| No. of pieces alive at 24 hours | 87 | 60 |
| No. of pieces reconstituting a head | 64 | 53 |
| Mean time required for head formation (days) | 2.3 | 4.1 |
| Head frequency (percentage) | 73.5 | 88.3 |

constituted at the cut end of the piece since resorption was impossible.

The chief factor preventing head formation in pieces which did not reconstitute appeared to be their short length; such pieces survived as long as was required for head formation in reconstituting pieces, but eventually died without any indication of reconstitution—none survived more than 7 days. Although the actual head frequency appears to be higher in worms from the laboratory stock, the rate at which reconstitution proceeds is indicated to be retarded as shown by the time required in the two sets. Figure 1 compares these data graphically. It is important to point out here that in more than one hundred and fifty specimens of *S. grande* sectioned, only one ever developed a fission plane while the piece was without a head.

## RECONSTITUTION OF S. TENUICAUDA

No account of the process of reconstitution in *S. tenuicauda* exists in the literature under that specific name, although the de-

scription of the structure and reconstitutional behavior given by Child (1903) for the species called by him *S. leucops* so closely approximates that observed in the present study in *S. tenuicauda* and differs so markedly from the present *S. leucops* as to suggest that Child described the reconstitution of *S. tenuicauda* under the name of *S. leucops*.[1] That the reconstitution of *S. tenuicauda* is essentially like that of *S. grande* is shown by the process of reduction and resorption diagrammed in the following figures. The case chosen as

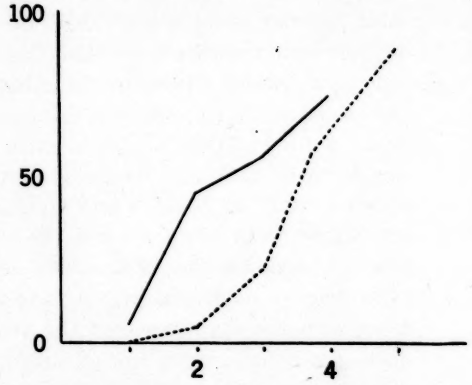



FIG. 1.—Graph comparing the relative rate of reconstitution of freshly collected and laboratory *Stenostomum grande*, the line —— indicating the former, and . . . the latter. The data are taken from Table I; the ordinates represent head frequency; the abscissae, the time (in days) after section at which the observations were made.

being a typical illustration of the process of reconstitution in *S. tenuicauda* is that of the worm shown in Figure 2, which was collected May 5 and operated on, as indicated, the following day. The chain was made up of five distinct zooids, with well-defined fission planes of the developmental sequence indicated by the figures to the left of each fission plane. Likewise the order of development of the zooids is indicated by the inclosed Arabic numerals, which, like the Roman numerals of the fission planes, follow the scheme devised by Child (1902) in his study of *Stenostomum*. By this system

[1] Professor Child has informed me that he now believes his identification of the species which he then called *S. leucops* to have been incorrect and that the form was probably *S. tenuicauda*.

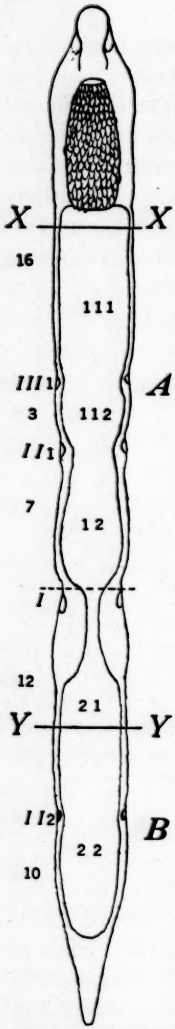
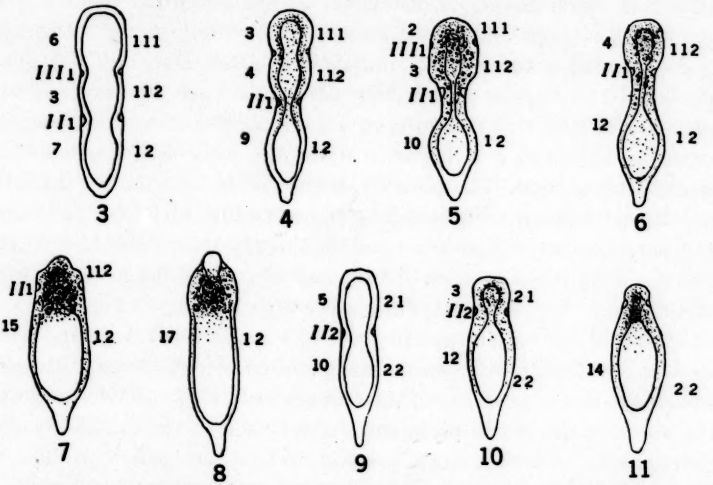



FIG. 2.—A freshly collected *Stenostomum tenuicauda* with five zooids, showing length and position of sections removed. Explanation of nomenclature in text

of nomenclature the two zooids of a chain of two are indicated by 1 and 2, respectively, 1 being the anterior and 2 the posterior; this chain gives rise to a chain of four zooids by division of each of these two, and the four are numbered, beginning at the anterior end of the chain, 1 1, 1 2, 2 1, 2 2. Similarly, when each of these zooids divides, the further history is indicated by adding another figure. Thus in the five-zooid chain figured, the most anterior zooid has divided once more than the posterior member 2, and this is shown by the figure 1 1 1 which represents the oldest member of the chain, and 1 1 2, which is the youngest zooid. Zooid 1 2 is the third oldest member in point of development and was originally a part of the anterior zooid 1. Zooid 2 has divided but once, and its posterior zooid 2 2 was the fourth to appear. The fission planes are also designated in their order of development, which of course coincides with the appearance of the zooids. In the development of a chain from a single zooid there are: one fission plane of the first order, separating zooids 1 and 2; two of the second order, separating zooids 1 1 and 1 2, and 2 1 and 2 2, respectively; and four of the third order in a chain of eight zooids; and so on. These orders are distinguished by the Roman numerals I, II, III, etc.; and their position in the chain is indicated by adding to the Roman numeral denoting its order an Arabic numeral denoting its position. Thus in the five-zooid worm shown in Figure 2, the first fission plane of the second order to arise was the anterior, which is indicated by II 1, and the first of the third order was also the anterior III 1. All observations indicate that this anteroposterior order of development is very closely adhered to in *Stenostomum;* this matter will be treated more fully in a later paper. In following descriptions and figures

of the development of sections taken from the chain, the fission planes retain the original number, thus indicating at once the position of the piece in the original chain. When new zooids appear in the cut piece, as in *S. leucops*, their relation to the original zooids is clearly shown by this system of nomenclature. The length of the zooids is indicated in all cases by figures in arbitrary micrometer




FIGS. 3–11.—Sketches taken at intervals to show course of reconstitution in pieces from the worm shown in Figure 2. Figures 3–8 show the process of resorption in the anterior piece *A*, and Figures 9–11 the same process in the posterior piece *B*.

units to the left of each zooid; thirty-four of these are equivalent to 1 mm.

The reconstitution of *S. tenuicauda* is illustrated by two pieces taken from a five-zooid worm, shown in Figure 2. The anterior piece *A* was cut transversely at the body-level *XX* and the worm induced to separate at fission plane I; it comprises the intestinal portion of 1 1 1 and the complete zooids, 1 1 2 and 1 2, of which 1 2 is the older. The posterior piece *B* was cut at the body-level *YY* and comprises the partial zooid 2 1 and the complete zooid 2 2. The regulatory behavior of the anterior piece is indicated by Figures 3–7, which were made at intervals after the operation.

Figure 3 shows the piece as it appeared 1½ hours after section. The cut end is closed and rounded off, and in the posterior zooid the

intestine has healed the break caused by separation. At 11 hours the form of the piece was essentially that found previously. Figure 4 shows the appearance of the piece at 22 hours; the anterior zooid has decreased very considerably in length, and the intestine anterior to II 1 is much smaller and partly obscured by masses of cellular material. The ciliated pits of 1 1 2 are distinct; while the development of 1 2 is much advanced, as shown by the condition of II 1, the narrowed anterior end of the intestine, and the increase in length. A distinct tail is now present, and cellular débris is seen in the pseudocoel of 1 2. Figure 5 (five hours after this stage) shows a further decrease in length of the anterior partial zooid and a continued increase in length of 1 2. Figure 6 shows the piece as it appeared 47 hours after section. The position of the ciliated pits of 1 1 2 could not be determined with certainty but appeared to lie not far from the anterior end. Zooid 1 1 1 has practically disappeared, and the results of its disintegration fill the pseudocoel of the anterior half of the piece. Six hours later the piece appeared as in Figure 7; no ciliated pits are evident except those of 1 2 which are large and well developed. The anterior width has increased as if the body wall were forced out by the products of the reduced anterior parts which crowd the anterior end of the piece and extend back to the tail of 1 2. A short portion of zooid 1 1 2 still remains to be resorbed, for the ciliated pits at II 1 are not in their normal position. That some head-modeling is occurring is indicated by the altered shape of the anterior end. Figure 8 shows the reconstituted worm with the ciliated pits in their proper position and a nearly normal head shape. The length of the new worm is approximately that of the piece with which the process started. No trace of the former narrow anterior portion of the intestine of 1 2 could be seen because of the obscuring mass of cellular débris. The process of reconstitution is essentially ended by the complete resorption of anterior parts, for the formation of a new mouth and pharynx and the complete disappearance of disintegration products from the pseudocoel of the resorbing zooid are of no interest here.

The process of reconstitution in the posterior piece *B* is followed in Figures 9–11. The first, showing the piece as it appeared 1½ hours after the operation, gives the initial length of the partial zooid 2 1

in which the cut anterior surface has assumed a rounded form. At 11 hours after section, a slight reduction was seen in the anterior piece, while II 2 had continued to develop, although there was no change in the length of 2 2. Figure 10 shows the piece at 22 hours, with the anterior piece distinctly reduced and the disintegration products crowding the anterior half of the entire piece. Zooid 2 2 has now increased in length, and the fission plane is well advanced. By 32 hours, resorption of 2 1 was complete, as shown in Figure 11. It is of interest that the process of resorption took less time for completion in the latter piece than in the anterior piece *A*. This is undoubtedly due to the smaller amount of tissue undergoing resorption in the case of *B*.

It is to be noted in the case of the piece *A* that not merely the posterior part of a single zooid was resorbed, as in *B*, but that a complete zooid plus the greater part of another has disappeared. Fission plane III 1, together with its developing ganglia, was completely destroyed in the course of reconstitution, with the result that the most posterior head region of the cut piece became the head region of the entire reconstituted piece. Child (1903) has previously called attention to the fact that the resorbing zooid is always the oldest and most highly developed of the zooids of the cut piece.

The description given above is typical of the result of reconstitution in pieces which are cut to include one or more fission planes. If, on the other hand, a piece be so cut from a chain that it includes no fission plane or is simply a single zooid of which the head has been removed, reconstitution of a head by resorption is impossible. In no observed case was a fission plane found to appear in such a single headless zooid, a fact which is of great interest when comparison is made with the situation in *S. leucops*. A new head is reconstituted in such pieces by the formation of a complete new head region at the cut anterior surface, of which the first indications are ciliated pits in their proper position on the anterolateral borders of the piece.

The comparative head frequencies of freshly collected and laboratory-cultured worms is shown in Table II. These data, like those of *S. grande*, compare the head-frequency of tail zooids of two- to five-zooid worms, none of which contained a fission plane, so that

reconstitution must take place by formation of a new head at the cut end of the piece and not by resorption. The laboratory stock had been cultured for 2 months. Here, again, it must be pointed out that a fission plane was never observed to develop in a cut piece while it was without a head.

Here, as in *S. grande*, the pieces which fail to reconstitute are chiefly short in length; after surviving in some instances as long as 6 days, such pieces disintegrate without showing any signs of reconstitution. That culturing induces a retardation in rate of reconstitution is not so clearly indicated as it was in *S. grande*. The two sets of data are shown graphically in Figure 12; included in the graph for comparison are the data from the reconstitution of thirty-

TABLE II

RECONSTITUTION OF FRESHLY COLLECTED AND LABORATORY-GROWN S. TENUICAUDA

| | Freshly Collected Material | Cultured Stock |
|---|---|---|
| No. of pieces alive at 24 hours after section | 46 | 39 |
| No. of pieces reconstituting a head | 39 | 34 |
| Mean time required for head formation (days) | 2.2 | 2.5 |
| Head frequency (percentage) | 80.4 | 87.2 |

eight anterior pieces of three-zooid cultured worms, of which thirty-seven reconstituted heads. Since each of these pieces consisted of one complete zooid and an anterior partial zooid, they reconstituted by resorption. In both *S. grande* and *S. tenuicauda* this form of reconstitution was found to require less time than the complete formation of a new head.

*Stenostomum grande* and *S. tenuicauda* have been shown to be similar in a number of respects: Both reconstitute readily by resorption in the presence of a fission plane, both show a high frequency of head reconstitution, and both are alike in having an extremely low frequency of fission-plane development in pieces without a head. An apparent exception to the last statement should be explained: In a chain of zooids with the head of the most anterior zooid removed, the posterior zooids may separate from the anterior zooids at the oldest fission plane in the chain, when this fission plane is so

far advanced that it is nearly ready to separate in the natural course of events. When the oldest zooid of the chain in the absence of the head of the chain is slightly less developed than in the preceding case, resorption of the anterior zooids or parts of zooids occurs; and as a result of the distribution of the reduced anterior materials, a posterior zooid may so increase in length and development that it separates from the resorbing zooid. In reconstitution by resorption, however, the dominant zooid of the piece which effects the actual reduction and resorption of the anterior parts is always in complete control of the entire piece, so that the appearance of a new posterior fission plane as a result of growth is as natural as the appearance of a new zooid in a chain with the dominant head region properly oriented. But in the second form of reconstitution in these two species, in which the cut piece is without a morphological head of any kind, a new fission plane very rarely makes its appearance until the new anterior head is advanced in its development. This behavior during reconstitution stands in the sharpest contrast with that to be described in *S. leucops*.

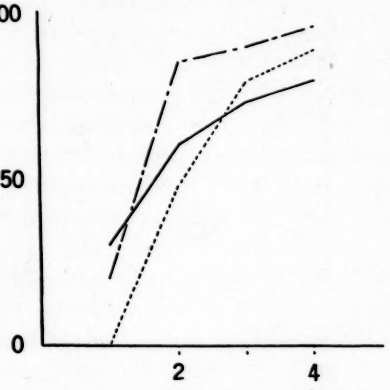



FIG. 12.—Graph comparing the relative rate of reconstitution of freshly collected and laboratory *Stenostomum tenuicauda*, the line —— indicating the former, the line – – – the latter, and the line —— – —— anterior zooids as explained in the text. The data are from Table II; the ordinates represent head frequency; the abscissae, the time (in days) after section at which the observations were made.

## RECONSTITUTION IN S. LEUCOPS

Work on the reconstitution of the species here identified as *S. leucops* had not proceeded far when it became apparent that the process differed radically from that described for the species of that name by previous investigators. The regulatory behavior after section of *S. leucops* from all available sources was marked by the

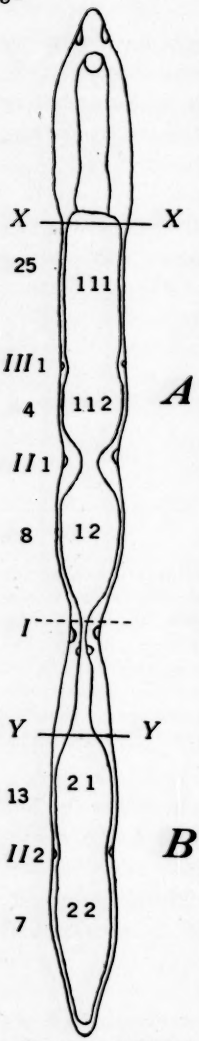



FIG. 13.—A *Stenostomum leucops* with five zooids grown in the laboratory, showing the position of the two pieces *A* and *B* used in the experiment.

absence of any process of resorption, which is all the more striking because of its great importance in the reconstitution of the two related species *S. grande* and *S. tenuicauda*. A further departure from the behavior of the other two species was found in the head frequency of cut pieces; it has been shown that the great majority of cut pieces of these two species reconstitute heads with great readiness and become normal individuals. On the contrary, in *S. leucops* the great majority of cut pieces do not reconstitute a head by any method, a situation which leads to the production of acephalic pieces showing no signs of reconstitution and which finally die after surviving many days.

The course of reconstitution in *S. leucops* is described by the following typical examples, the first of which shows the more common lack of any degree of head reconstitution, and the second a case in which a head is reconstituted.

Figure 13 illustrates a typical *S. leucops* of five zooids taken from a stock which had been cultured at room temperature for 43 days. Zooids, fission planes, and length are indicated as previously described. The pieces *A* and *B* employed for experiment were removed by cuts at the body-levels *XX* and *YY*, *A* being separated posteriorly at the fission plane I. Piece *A* comprises the intestinal portion of 1 1 1, and all of zooids 1 1 2 and 1 2, and thus includes two fission planes. Piece *B* comprises the partial zooid 2 1 and all of 2 2, including fission plane II 2. It will be noticed that these pieces are exactly similar to those employed in the experiment with *S. tenuicauda*. The regulatory behavior of the two pieces is illustrated by the observations taken at intervals after section.

Figure 14 shows piece *A* 1½ hours after the operation. As after all such cuts, the open wound is closed by a contraction of the circular musculature of the body wall, and the cut end assumes a rounded form which persists as long as the piece survives unless reconstitution of a head occurs. The intestine also heals quickly and rounds off; the intestine and body wall are separated by a clear area in which strands of parenchyma appear. No further change at this cut end occurs unless ganglia and their associated ciliated pits develop.

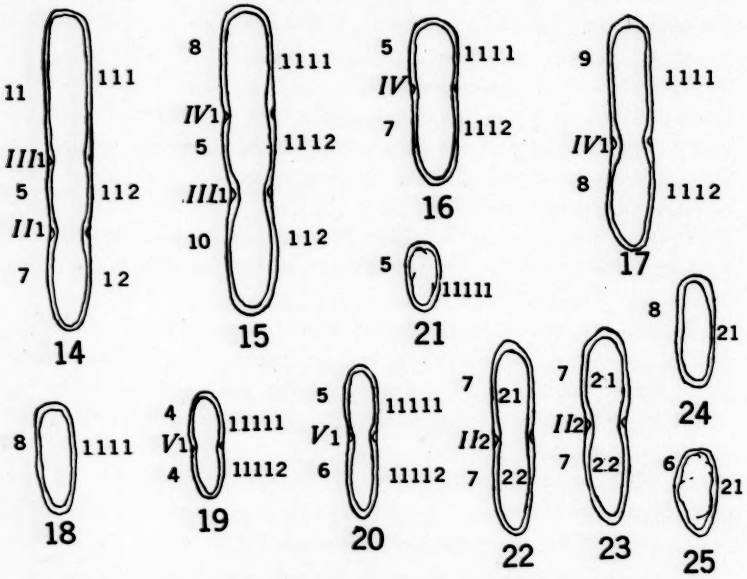
In Figure 15 the piece is shown as it appeared 22 hours after the operation. Zooid 1 1 1 has divided into two, 1 1 1 1 and 1 1 1 2; and zooid 1 1 2 is now the posterior zooid of the piece. Zooid 1 2 has completely separated from the cut piece and was found as a normal individual free-swimming in the dish. All zooids of the piece show an increase in length, which presumably is at the expense of food material present in the intestine at section as well as from the food reserves of the body.

Figure 16 shows the piece 31¼ hours after section. Only two zooids remain of the cut piece and represent the original partial zooid 1 1 1. Zooid 1 1 2 has separated and is a normal individual. There is no indication whatsoever of reconstitution at the cut end of the piece remaining.

By 46 hours after section, the two remaining zooids of the cut piece appear as shown in Figure 17. Both have increased in length, and 1 1 1 2 seems to be preparing to separate from 1 1 1 1. At an observation taken 71¼ hours after section, this separation has occurred and all that remains of the original cut piece is shown in Figure 18. By 94 hours, this piece has divided for the second time after being cut (Fig. 19); and 24 hours later, or 118 hours after section, an increase in the length of each of the two zooids is apparent (Fig. 20). At 142 hours after section, zooid 1 1 1 1 2 was found to have separated, and Figure 21 shows the last remaining bit of the original cut piece as a mass of tissue in which endoderm and body wall are still distinct but with absolutely no indications of reconstitution. At 170 hours after section, the piece was found disintegrating.

Piece *B* shows the same behavior and absence of any indication of head reconstitution at the cut end (Figs. 22–25). Figure 22 shows

the cut piece as it appears 1½ hours after section, consisting of the partial zooid 2 1 and the complete zooid 2 2. At 22 hours (Fig. 23), the appearance is the same save that 2 2 has continued to develop its head region. By 46 hours, 2 2 has separated, leaving 2 1, as shown in Figure 24. No further change takes place in the remaining partial zooid; it gradually decreases in size, appearing at 142 hours after




FIGS. 14–25.—Sketches of the cut pieces *A* and *B* made at intervals after section as shown in Figure 13. Figures 14–21 show the absence of head reconstitution at the anterior cut end of the piece *A* and the formation and subsequent separation of posterior zooids in the cut piece. The normal zooids separating from the piece are not shown.

section as in Figure 25, and was found dead at 170 hours, having shown no indication of reconstitution after being cut.

Other similar examples of cut pieces have shown the anterior partial zooid to survive as long as 24 days with no signs of reconstitution during this time. Such cut pieces lie quietly on the bottom of the watch glass or creep about very slowly by ciliary movement. The movements do not suggest to any degree the co-ordinated re-

actions of the normal worm; there is no reason to suspect, from microscopic study or otherwise, that the anterior ends of such pieces have undergone the slightest degree of reconstitution of a new head region.

Pieces of *S. leucops* do, however, develop heads in some cases. Figure 26 shows a chain of four zooids collected August 27, 1927, and cut 2 days later at the body-levels *XX* and *YY* after induced separation at the fission plane I. The anterior piece comprises the intestinal portion of zooid 1 1 and the complete zooid 1 2. Piece *B* consists of the partial zooid 2 1 and all of zooid 2 2, and each piece includes a well-developed fission plane.

Piece *A* appeared at 19½ hours as shown in Figure 27. Both zooids have greatly increased in length, especially the posterior. Forty-three hours after section (Fig. 28), zooid 1 2 has separated from the anterior member, which has meanwhile divided by a new fission plane. Figure 29 shows these two zooids as they appeared 67 hours after section. Both have increased in length, and the new fission plane is well advanced in development. From very faint indications of ciliated pits in their proper position on the anterior zooid and the directed swimming movements of the piece as a whole, it appears very likely that a head is being reconstituted at the anterior cut surface. Figure 30 shows the piece at 91 hours after section. The posterior zooid has separated. It is now clear that the anterior piece is reconstituting a head —the ciliated pits are clearly visible, the former rounded anterior end is changing, the anterior end of the intestine is obscured by a mass of cells but has disappeared for some distance anteriorly; the piece swims about actively. At 121 hours after section (Fig. 31), the head form

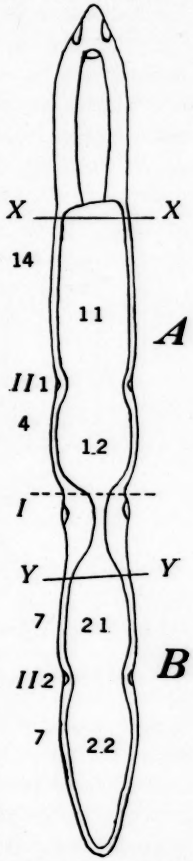
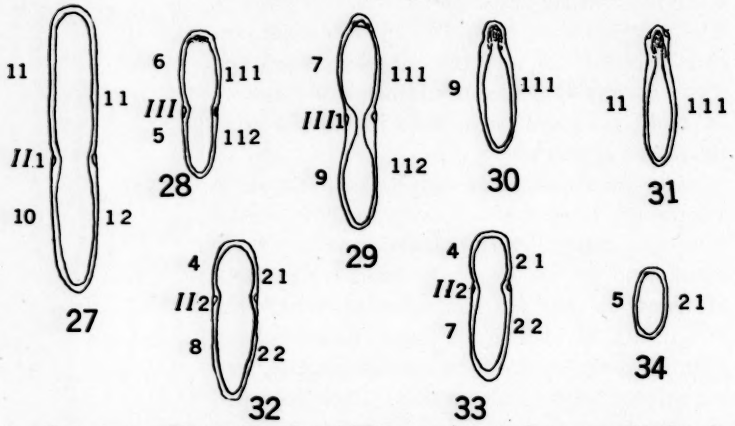



FIG. 26.—A freshly collected *Stenostomum leucops* with four zooids, showing the position of the two pieces *A* and *B*.

is nearly normal, and a pharynx is forming. Twenty-four hours later, the former headless piece was practically indistinguishable from a one-zooid individual formed by fission.

Piece *B* is shown in Figure 32 as it appeared 19 hours after section; Figure 33, 24 hours later, shows the fission plane better developed but with no change in the anterior piece. At 67 hours after section (Fig. 34), zooid 2 2 has separated from the anterior member of the piece. By 91 hours, the anterior piece has decreased in size




FIGS. 27–34.—The anterior piece *A* (Figs. 27–31) reconstitutes a new head at the anterior cut end of the piece with no resorption occurring. Figures 32–34 show the absence of head reconstitution in the posterior piece *B*.

until it is hardly more than a circular mass of tissue, which disintegrated within 24 hours.

In all cut pieces of *S. leucops* which reconstituted a head, the only process of reconstitution found was that in which the head formed *de novo* at the anterior cut surface as described above. The only observations that might be interpreted as possible abortive attempts at reconstitution by resorption were a few cases in which measurement of length of zooids suggested, with no degree of certainty, that a posterior complete zooid was increasing in length at the expense of the anterior partial zooid. The absolute increase seen in the length of all zooids of a cut piece, however, obscured any possible significance of relative increase in the different zooids. In no

case was material from the anterior parts of the piece found in the pseudocoel of the posterior zooid, though this is a conspicuous feature in resorption in the other two species.

The foregoing description of reconstitution in *S. leucops* was illustrated by the behavior of cut pieces of four- and five-zooid worms for the sake of comparison with similar cut pieces of the other two species. Exactly the same process could be shown with pieces taken from one-zooid worms or from eight-zooid chains, or with pieces including fission planes in any stage of development. Because they were more plentiful in the laboratory cultures and could easily be cut to yield pieces either with or without a fission plane, three-zooid worms were ordinarily used in the work on reconstitution under ex-

TABLE III

RECONSTITUTION OF FRESHLY COLLECTED S. LEUCOPS

| | Tail Zooids | Anterior Zooids |
|---|---|---|
| No. of pieces cut | 64 | 25 |
| No. of pieces reconstituting head | 14 | 14 |
| Head frequency (percentage) | 21.9 | 56 |

perimental conditions. The posterior tail zooid was favored because, in using it, failure of reconstitution at the only cut surface could not be interpreted as inhibited by stimuli arising at the posterior cut surface.

The head frequency of cut pieces of freshly collected *S. leucops* is shown in Table III. The pieces described as tail zooids formed the posterior zooid of chains of two- to five-zooid worms; none of them included a visible fission plane at the time of section. The anterior pieces consisted of the intestinal region of the most anterior zooid of the chain and either one or two complete zooids posterior to this. In each instance in which the cut piece reconstituted, the new head was formed at the anterior cut end. Pieces that died within 24 hours of section are not included in the number of pieces listed as cut.

It is particularly apparent in the tail zooids that not only is the number of pieces reconstituting heads distinctly lower than is the rule in *S. grande* and *S. tenuicauda* but the time required for head

formation is much longer than in the other species. Figure 35 brings out this difference graphically; the amount and rate of reconstitution in comparable tail-zooid pieces from freshly collected worms of the three species is compared. None of the pieces contained fission planes, so that reconstitution could only occur by formation of a new head.

### RECONSTITUTION AND FISSION IN S. LEUCOPS IN VARIOUS CULTURE MEDIA

The extremely low proportion of cut pieces of *S. leucops* in which a head was reconstituted, which stood in striking contrast with the behavior of similar pieces of the other two species of *Stenostomum*,

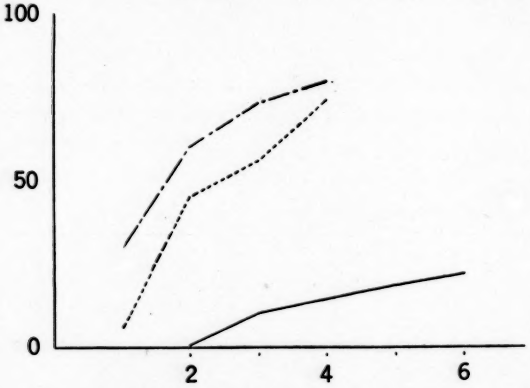



FIG. 35.—Graph comparing the relative rate of reconstitution in tail-zooid pieces from three species of freshly collected *Stenostomum*. The ordinates represent head frequency; the abscissae, the time (in days) after section at which the observations were made. The line — — — indicates *S. tenuicauda;* the line – – –, *S. grande;* and ——, *S. leucops*.

led to attempts to localize a possible factor or factors in the external environment of the worms inhibiting reconstitution. Environmental changes with pieces from freshly collected material must necessarily be limited to post-operative conditions, and for this purpose cut pieces were transferred to a variety of media, such as lake water, pond and lagoon water from which the worms were taken, untreated well water, and the standard treated well water. None of these were of any advantage as regards head frequency. The laboratory stocks

afforded wider opportunity for changing environmental conditions. Cultures were grown in protozoan cultures made up in hay infusion, boiled cabbage and lettuce leaves, boiled *Elodea*, beef extract, and wheat. The worms were introduced into low concentrations of beef extract, malted milk, casein, compressed yeast, and boiled *Elodea* in which no protozoa were present. Small numbers of worms were fed with a superabundance of finely ground and washed beef liver, compressed yeast, and thick *Paramecium* suspension in which the ciliates had been injured by squirting them violently from a fine pipette. The standard wheat cultures were grown not only in treated well water and pond water but in dilute salt solutions. The cut pieces from standard cultures were transferred to media in which the pH had been adjusted to values from 6.2 to 8.6, to water with an $O_2$ content of 18 cc. per liter as compared with the ordinary 4.5 cc. per liter, to media of a range of osmotic pressure both hypertonic and hypotonic, and to water taken from cultures in which worms were rapidly multiplying by fission. The effects of narcotics, cyanide, and of changing the temperature conditions will be discussed separately. None of the conditions described showed any significant beneficial effect on the cut pieces as regards head frequency; some, of course, were distinctly injurious in that the pieces survived only a few days. Under ordinary conditions the pieces survived from 10 to 15 days, or longer with exceptional cases.

Table IV records the results from a number of sets of cut pieces subjected to a variety of treatment. All pieces were approximately of the same stage of development, being taken from the tail zooids of three-zooid worms and cut to include as much of the zooid as possible exclusive of the head region. Unless otherwise stated, the pieces are from worms grown under standard conditions. It should be noted that sets of pieces grown and cut under standard procedure show the same proportion of head reconstitution as do pieces experimentally treated. It is believed that the numbers concerned are large enough to give a true estimate of the reconstitutive capacity of the tail zooid grown under laboratory conditions.

That only 6 per cent of the pieces show head reconstitution (Table IV) as compared with 22 per cent of similar pieces from freshly collected worms (Table III) indicates that laboratory cul-

tural conditions are unfavorable for reconstitution. Evidence from the relative length of zooids, which will be treated in a later paper, indicates that it is the metabolic rate of the laboratory worm that is directly affected by the cultural conditions and that this, in turn,

TABLE IV

RECONSTITUTION OF TAIL ZOOIDS OF S. LEUCOPS GROWN IN THE LABORATORY UNDER STANDARD AND MODIFIED CONDITIONS

| Exp. No. | No. of Pieces | No. of Reconstructed Heads | Treatment |
|---|---|---|---|
| 260 | 10 | 1 | Grown and cut under standard conditions |
| 151 | 20 | 0 | As above |
| 261*b* | 10 | 1 | As above |
| 256 | 20 | 3 | Placed for 24 hrs. after section at 11° |
| 236 | 10 | 0 | Grown on yeast at 11°–18° after section |
| 233 and 241 | 30 | 2 | Fed for 7 days on yeast |
| 240 | 20 | 3 | Grown on yeast, cut into water of 18 cc. $O_2$ per l. |
| 239 | 20 | 2 | Grown in filtered lagoon water, cut into same as 240 |
| 235 and 218 | 20 | 1 | Grown and cut in ordinary lagoon water |
| 228 | 10 | 0 | Fed on beef liver |
| 213 | 10 | 0 | Hay-infusion protozoan culture |
| 183 | 10 | 0 | Cut into water from rapidly multiplying culture |
| 180 | 10 | 0 | Cut into water buffered to pH 8.6 |
| 176 | 10 | 0 | Grown on malted milk |
| 168 | 10 | 0 | Well water concentrated 4× by boiling |
| 167 | 10 | 0 | $\frac{1}{10}$ Ringer-Locke's solution |
| 150 | 20 | 0 | $\frac{1}{12}$ Ringer-Locke's solution |
| 165 | 10 | 1 | Cut into water of pH 7.4 |
| 163 | 20 | 2 | Cut into water of pH 7.2, and kept at 26° |
| 160 | 10 | 1 | Standard conditions except kept at 26° after section |
| 162 and 170 | 20 | 0 | Worms from 3-month old culture of nearly exhausted food supply |
| 161 | 10 | 0 | Cut into water of pH 6.2 |
| 158 | 10 | 0 | Standard culture grown at 26°–18° after section |
| 166 | 10 | 2 | Worms found in *Amoeba* culture from another laboratory |
| Totals | 340 | 19 | Head frequency = 5.6 per cent |

is reflected in the lowered amount and rate of reconstitution. Whether or not the conditions making for higher head frequency in worms taken from the natural environment are reproducible in the laboratory, the fact that new heads appear with regularity at fission planes in the growing whole worm even in cultures but appear only rarely at the anterior cut surface when the same worm is

sectioned is of great interest; but for the present, attempts at interpretation must be limited to tentative suggestions (pp. 54–55).

### EXPERIMENTS WITH CHEMICAL AGENTS

Attempts to alter head frequency in pieces of *S. leucops* by chemical agents found to be effective for *Planaria*, viz., anesthetics (Buchanan, 1923), KCN (Child, 1916), caffein (Hinrichs, 1924), and also by strychnin sulphate were unsuccessful. Table V gives the agents,

TABLE V

CHEMICAL TREATMENT USED IN ATTEMPTS TO ALTER HEAD FREQUENCY IN S. LEUCOPS

| Agent | Concentration | Exposure |
|---|---|---|
| Chloretone | m/350 | 1–2 hrs. before section, 3–5 hrs. after, followed by recovery |
| | m/200 | 45 min. before section, 1 hr. after, with recovery |
| | m/1,000 | Cut into solution and left there permanently |
| KCN | m/100,000 | 30 min. before section, 2½ hrs. and 20 hrs. after, with recovery. Also permanent exposure without renewal of solution |
| | m/12,800 | Cut into solution permanently but without renewal |
| | m/250 | 15–30 min. before section, 10 min. after, with recovery |
| Ethyl urethane | 1 per cent | 30–60 min. before section, 30 min. after, with recovery |
| Chloral hydrate | m/825 | Cut into solution, changed to fresh water 24 hrs. later |
| Caffein | m/80 | Cut into solution, changed to fresh water after 3 hrs. |
| Strychnin sulphate | 0.1 per cent | 6–50 min. before section |

concentrations, and exposures used in these experiments, the solutions being made up in treated well water, like that in which the pieces recovered. In none of the agents used were the worms or pieces completely immobilized, even in concentrations or exposures that produce cytolysis.

### FISSION IN HEADLESS PIECES

It was noted in the foregoing description of the process of reconstitution in *S. leucops* that cut pieces of this form, in contradistinction to those of the other two species, continue to divide by fission when the head of the chain is removed. This relation of fission to the

presence of the head of the chain is shown in the following experiment: Forty *S. leucops*, each with only two zooids and all of the same approximate length, were picked from a stock culture which had been started 3 weeks previously from freshly collected worms. The posterior zooids of all the worms were removed, and the heads of half the lot. Two sets of twenty pieces each were thus obtained: one set of zooids with heads in place, and a contrasting set with the heads removed. The appearance of the pieces at the end of 25 hours has been tabulated in Table VI. There would seem to be no doubt that the appearance of fission planes in operated pieces of *S. leucops* is directly dependent on the removal of the dominant head region of the zooid, which permits the development of heads in regions

TABLE VI

INFLUENCE OF HEAD ON FORMATION OF NEW FISSION PLANES IN S. LEUCOPS

| | With Heads | Without Heads |
|---|---|---|
| No. of zooids in set | 20 | 20 |
| No. of zooids with fission plane at start | 0 | 0 |
| No. of zooids with fission plane at end of 25 hours | 2 | 16 |

where such development was previously inhibited by their nearness to the dominating head region.

The hypothesis advanced to explain the failure of a high percentage of cut pieces of *S. leucops* to reconstitute heads concerns the length of the cut piece. It was observed that the piece which later reconstituted was noticeably longer than those which did not. The short length was usually brought about by fission, which left the anterior part of the piece too short to reconstitute a head. Table VII lists the sets of Table IV in which reconstituting worms appeared, and compares the length of the worms which later reconstituted as measured at the first observation with the mean length of all other pieces of the set. In practically every case the worm which later reconstituted a head was appreciably longer than the other worms of the set immediately after cutting. Other worms of equal superiority as regards length lost this advantage through the development of fission planes; this is very evident in the last set.

In order to test this hypothesis experimentally, pieces were cut as long as possible: the longest zooid of the chain is invariably the first, which is also the oldest and best developed. By making the anterior cut as far anterior as could be done without running the risk of including the posterior ends of the ganglionic masses in the piece and the posterior level the fission plane of the next successive zooid, a piece almost twice the ordinary length could be obtained. It had previously been shown that the pharynx and head alone

TABLE VII

RELATION OF LENGTH OF PIECE TO RECONSTITUTION AND FISSION
(Data from Table IV)

| Exp. No. | No. of Pieces | No. of Pieces Reconstituted | Length of Reconstituted Pieces (Micr. Units) | Mean Length of Others (Micr. Units) | No. of Pieces Forming Fission Planes |
|---|---|---|---|---|---|
| 260..... | 10 | 1 | 13 | 7.6 | 4 |
| 256..... | 20 | 3 | 10–9–12 | 9.3 | 6 |
| 241..... | 20 | 1 | 13 | 4.9 | 3 |
| 240..... | 20 | 3 | 10–11–10 | 4.8 | 2 |
| 239..... | 20 | 2 | 9–10 | 6.0 | 0 |
| 235..... | 10 | 1 | 6 | 6.0 | 0 |
| 234..... | 20 | 1 | 10 | 6.8 | 2 |
| 233..... | 10 | 1 | 11 | 7.2 | 0 |
| 163..... | 20 | 2 | 9–11 | 5.8 | 0 |
| 166..... | 10 | 2 | 8–10 | 11.5 | 8 |

quickly disintegrated, but that if a small amount of intestinal region were included in the piece, the piece reconstituted its normal relationships. In the absence of the head, pharynx plus intestinal region occasionally reconstituted a new head. Advantage was next taken of the fact that worms, after a period of some months' culturing in the laboratory, no longer formed fission planes with readiness following removal of the head. Figure 36 shows the two types of pieces cut, both having the same approximate anterior level, but the one only about half the length of the other. Table VIII shows the success attained by the use of this technique. The mean length of the long pieces (*l*) was 12.2, and that of the short ones (*a*), with the same anterior level, was 6.5; although of the fifty originally cut, seven died within 2 days after cutting, presumably because they contained too little intestinal region or none at all. Only three pieces, all long, divided by fission while in the headless condition. The fact

that an average time of 7 days was required for head formation indicates that though the amount of reconstitution may be increased by this method, the rate at which the process occurs is not increased. Only twenty of the short posterior pieces, (*b*) in Figure 36, were saved, and none of these had reconstituted by 6 days. The cultures shown in Table VIII represent subcultures of original cultures started about the first of October from freshly collected worms.

In old laboratory stocks growth is less rapid and the lengths of zooids are greater than in freshly collected animals. With anterior pieces of such animals cut as long as possible without including fission planes and subjected to low temperatures, the head frequency approximated that of freshly collected anterior pieces. Two sets of long pieces from three-zooid worms taken from the foregoing cultures were used, the anterior set like those shown in Figure 36, and the posterior set the tail zooid of the same worm cut anteriorly as close to the ganglia as possible. Both sets were placed at once at 8°C. and left there for 68 hours, after which they were brought back to room temperature. Fifty per cent of the anterior pieces reconstituted heads and 20 per cent of the tail pieces, which compares very favorably with freshly collected material. The heads were very slow in appearing, and developed in the posterior pieces before they did in the anterior—within 9 days in the former and between 12 and 15 days in the latter.

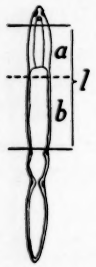



FIG. 36.—Figure showing the position and relative length of pieces mentioned in the text.

The reverse procedure of subjecting the pieces to high temperature immediately after section met with no success as regards increased head frequency. Worms from cultures grown at 11°, 15°, and at room temperature were placed at temperatures of 26° and 33° following section, and though the increase in length found in cut pieces on removal from the chain and development of fission planes took place sooner than in pieces kept at room temperature, no consistent increase in head frequency could be demonstrated.

### ADVENTITIOUS HEADS

A phenomenon discovered accidently threw considerable light upon the head-forming potentialities of various body-levels of *S.*

*leucops*. Worms with four or five zooids to the chain were placed in a Syracuse watch glass containing water to a depth of 2–4 mm. and were exposed for $4\frac{1}{2}$ minutes to unfiltered radiation from a Cooper-Hewitt quartz mercury arc lamp. The source of radiation was approximately 44 cm. from the material under treatment. The voltage was about 80; and the amperage $3\frac{1}{2}$–$4\frac{1}{2}$, direct current. Following this radiation, the worms were washed twice in fresh water and left in a third dish of water. Within 5 hours after radiation, the zooids

TABLE VIII

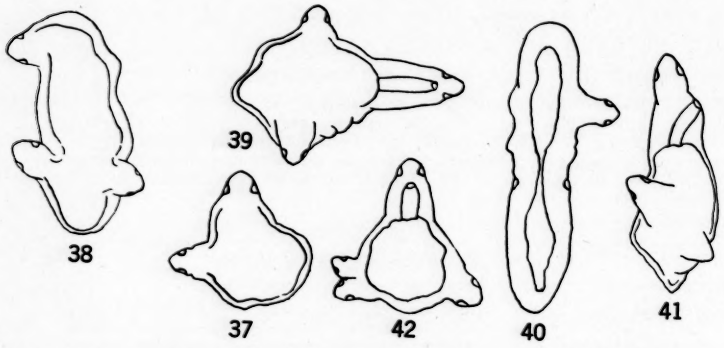
| No. of Pieces Cut | No. Dying | No. Reconstituted | Cultural Age |
|---|---|---|---|
| | | Long Pieces* (*l* in Fig. 36) | |
| 10 | 0 | 4 | Cut December 20, worms from 18-day-old culture |
| 10 | 0 | 4 | Cut January 14, worms from 24-day-old culture |
| 10 | 0 | 3 | Cut January 24, worms from 53-day-old culture |
| 20 | 0 | 6 | Cut February 22, worms from a number of cultures |
| 50 | | 17 | |
| | | Short Pieces† (*a* in Fig. 36) | |
| 10 | 1 | 2 | Cut December 20, worms from 18-day-old culture |
| 10 | 2 | 0 | Cut January 14, worms from 24-day-old culture |
| 10 | 1 | 1 | Cut January 24, worms from 53-day-old culture |
| 20 | 3 | 2 | Cut February 22, worms from a number of cultures |
| 50 | 7 | 5 | |

* Head frequency = 34 per cent.
† Head frequency = 11.6 per cent.

broke apart with considerable disintegration occurring at the fission planes; and from 24 to 48 hours after the treatment head-like outgrowths began to appear on these separate zooids, which in many cases developed distinct ciliated pits. In most pieces the original head persisted in addition to the supernumerary heads, but in others a single new head appeared to establish a new polarity in the zooid. The multipolar forms showed as many as four distinct heads on what was originally a single zooid. None of the forms survived longer than 7 days. Although these results were duplicated with other chains of *S. leucops*, it could not be done with *S. grande* or *S. tenuicauda*. Figures 37–42 show some of the forms obtained with *S. leucops*.

It seems probable that such adventitious heads arise as a result,

first, of the obliteration of the original gradient in the zooid (as Hinrichs [1926] has demonstrated for other forms), and second, of the origin of new polarities as gradients in relation to regions of greater activity, fortuitously or otherwise localized. When the old gradient is not completely obliterated (e.g., in zooids in which the old head persists), its control over the remainder of the piece is so reduced that heretofore suppressed regions develop as independent dominant centers upon recovery. Attempts were made to duplicate these effects obtained with ultra-violet radiation by the use of KCN.




FIGS. 37–42.—Adventitious or supernumerary heads produced in *S. leucops* by means of ultra-violet radiation. The original or persisting head of the zooid is indicated by the mouth and pharynx.

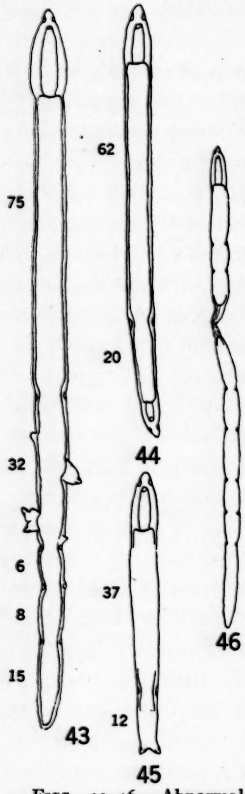
Chains of zooids, exactly like controls which produced adventitious heads following radiation, were exposed to concentrations of KCN from m/200 to m/1,000 for various periods, and then allowed to recover in fresh water, but no indications of adventitious heads appeared in any case.

A similar abnormal outcropping of heads had previously been found in certain experimental cultures, this time on the whole worm. On December 8, 1926, a culture of *S. leucops* had been started for another purpose which involved adding 100 mg. of twice-sublimated iodin crystals to 1 liter of treated well water with wheat and protozoa as the food basis. Forty-two days later many abnormally long worms were found in this culture, which showed derangement

of normal zooid succession and more or less haphazard outcropping of supernumerary heads from the body wall. Most of the original iodin had escaped from the water long before, no yellow color and only a faint iodin odor remaining.

At the same time the old iodin culture was examined, an even larger number of abnormal worms were found in old experimental cultures which had originally contained KCN in low concentrations. These cultures had been started two months previously with four concentrations of KCN, m/50,000, m/100,000, m/200,000, and m/500,000, with a control in well water like that to which the KCN had been added. Two grains of wheat and protozoa had been added at once to each 150 cc. finger-bowl culture, and two more grains one month later. This represented the total food supply of the cultures during the 2 months. The cultures were examined every other day during the first 2 weeks, but examination was discontinued when it was seen that the worms in each dish survived and multiplied. During this initial period, it was noticed that the highest concentration of KCN retarded the multiplication of worms in that culture, and the number of worms progressively increased down to the m/200,000 concentration, in and below which the KCN was apparently too weak to have any noticeable effect. At the time of discovery of the abnormal worms, this difference in the number present was not apparent; all KCN had decomposed and volatilized long before. However, the worms in the m/50,000 culture showed a higher proportion of abnormalities than the other cultures. The control showed no abnormalities at the time the experimental cultures did, but 3 weeks later, similar forms were found in it, indicating that although the depressing agent may have had a predisposing effect, the actual causative factors must lie in the cultural conditions themselves. Other evidence indicates that scarcity of food may have increased zooid length. Although the experimental conditions of the KCN and iodin cultures have been repeatedly duplicated and both whole animals and pieces have been subjected to a wide range of concentrations of KCN in experiments for other purposes, no adventitious heads have ever been obtained experimentally in any case. Figures 43–46 illustrate a few of the abnormal forms found in the KCN cultures. Most striking perhaps are Figures 44 and 45,

in each of which a head has formed at the extreme posterior end of the worm and points in the opposite direction from the normal head.




FIGS. 43–46.—Abnormal worms showing enormous length of zooid, irregular zooid succession, and adventitious heads arising from the body wall. Figures 44 and 45 show heads at the posterior ends of zooids and pointing in the opposite direction of the original gradient.

A number of pieces were cut from different body-levels of such abnormally long-zooided worms, but none of them were found capable of reconstituting a new head at the cut end. Fission planes developed readily in such pieces, with normal worms separating. Cut pieces which included an adventitious head died within 10 days without any great changes in condition. The head, though possessing ciliated pits and showing independent muscular contractions and movements, was apparently unable to take over complete control of the rest of the piece. Occasionally when the cephalic outgrowth was long enough to include an intestinal region, it dragged about in an unwieldly way the very much larger mass of the piece from which it arose. In no observed instance, however, did a head resorb the rest of the piece, or bring it into normal relations with itself. Usually the original anterior end of the piece, in spite of the absence of a head, dominated the rest of the piece, moving forward at times despite independent muscular movements of the adventitious head which was unable to attach itself to the substratum.

### INFLUENCE OF SEXUALITY ON RECONSTITUTION AND FISSION IN STENOSTOMUM

Worms of the genus *Stenostomum* are described in the literature as "protandric hermaphrodites." During the greater part of the year they reproduce asexually by fission; but in the late summer the male phase matures with the appearance of an ovoid, unpaired

testis dorsal to the pharynx in each zooid of the chain. Soon after the development and degeneration of the male sexual organs, ovaries appear ventral to the intestine, varying in number from one to six, and each containing from three to six ova in different stages of development. The female phase is characterized by the loss of fission ability, so that each female individual consists of a single large zooid of great length. According to Voigt (1894), each chain of zooids in the mature male stage of development breaks up into its component parts on the appearance of the female gonads. No one has recognized the female sex opening; the egg goes through the early cleavage stages within the body of the mother, where it becomes surrounded by a clear, transparent membrane, and is ordinarily stated to be set free by the death of the mother. Voigt, however, states that the female does not die but begins again to form zooids on the disappearance of the ovaries.

During the present investigation on *Stenostomum*, females of *S. grande* were found abundantly in ponds and lagoons during the month of October and early November. A few females of *S. tenuicauda* were also found at the same time. The male form was not found in any species, but Dr. Child has very kindly permitted me to make use of unpublished observations of his own made on males of *S. grande* in 1902–3. These notes explain why no males have been found in nature during the present study, since they show that the male phase matures in September, during which month the writer was absent from the city. No sexual stages of *S. leucops* were found at any time, so that the present discussion will be confined principally to *S. grande*.

The presence of testes apparently has no effect on the process of reconstitution, for, according to Child's unpublishd observations on male *S. grande*, reconstitution by resorption occurs under the proper conditions as readily in pieces taken from males as in those from asexual worms. Neither does reconstitution interfere with the development of the gonads. Testes do not appear simultaneously in all zooids of the chain, but first in the most anterior or oldest zooid, and later in the younger zooids. If a cut piece be taken from a worm in which only the anterior zooid has developed a testis, the cut piece is capable of developing a testis at the same time the zooid is resorb-

ing a partial anterior part. *Stenostomum grande* does not form new fission planes when the anterior end of the asexual chain is removed, and the male chain shows the same behavior. Child's observation confirmed the statement of Voigt that the zooids of a chain separate on the degeneration of the testes and become single female zooids. The ovary may appear in the oldest zooid before this separation has occurred. The results of sectioning a worm in such an intermediate sexual stage indicate that if the cut piece does not contain the very young ovary it is able to reconstitute a head. The piece containing the early ovary fails to reconstitute, as do completely female pieces from individuals, although the anterior zooid in which the ovary was developing may also contain a testis. This indicates the fact that is later proved by the failure of completely female worms to reconstitute a head under any circumstances, namely, that with the onset of femaleness in the individual, a physiological change in the reaction system is brought about which prevents head reconstitution.

*Stenostomum grande* taken from the lagoons during October show a high proportion of worms in the female stage of sexual maturity; of one collection of sixty-seven worms, fifty-five were females, the others asexual chains. The appearance of ovaries is correlated with a striking change in reconstitutional reaction. The readiness with which pieces from asexual chains reconstitute a head has been described above. But when ovaries are present in the worm, as few as one or as many as four, the worm loses all power of head reconstitution from any level posterior to the cephalic ganglia, independent of the length of the cut piece, the body-level from which it was taken, or whether the piece actually contained the gonad or was merely a part of the sexual zooid. If the head of such a typical long female individual is removed by a cut across the pharynx and the remaining pieces are divided into anterior and posterior halves, the anterior piece commonly disintegrates within 4 days, with no indication of head reconstitution. The anterior end becomes rounded and maintains that shape until death; the posterior end forms a short tail. The egg, if of any size at the time of section, continues to develop and is set free by the disintegration of the piece. The posterior piece of the original worm usually survives longer than the

anterior, occasionally living for 12–15 days. During this time, however, no signs of head reconstitution were ever found at the cut end. The head itself quickly disintegrates unless a small amount of intestinal tissue is included in the piece, in which case a new tail is formed and the worm, if fed, appears normal. Pieces of female individuals of less than half the intestinal length, as well as those in which merely the head is removed, also failed to reconstitute a head.

Observations on unoperated females showed that the egg was extruded from the body of the mother by the rupture of the body wall, the wound ordinarily closing again and the mother surviving for a few days but eventually dying. In some cases the rupture was so extensive or so severe that the worms died at once. Of some twenty worms with eggs kept under observation for some time, all died after voiding the egg; none regained the power of fission, as Voigt stated. Two of the eggs which were kept after extrusion developed as small worms in 25 days. They had been kept at room temperature during this time; under natural winter conditions the eggs probably do not develop until spring.

Repeated efforts were made during the course of this work to induce experimentally the appearance of sexuality in *S. grande* and *S. leucops*. The methods used were to feed the worms heavily with thick *Paramecium* suspension or with ground beef liver, and then to lower the temperature of the culture to 15°, 11°, 8° or 4°C. both directly and gradually through these successive temperatures to 4°. None of the attempts were successful. The reverse prodecure of raising the temperature to 26° and 32° was also tried but was equally unsuccessful. Without any attempt to induce their appearance, females of *S. grande* were found in the two ordinary laboratory cultures of this species during the months of November and December, 1927, and the first half of January, 1928. This is the only winter during which cultures of this species were kept in the laboratory. One of these cultures was started June 18 as a subculture to another started April 24 with freshly-collected worms. The other was started the last week in August from fresh material. Both were protozoan wheat cultures and were kept continuously at room temperature. No differences in appearance or in reconstitutional behavior between these females and those taken from the lagoons in the autumn could

be seen. The male stage undoubtedly preceded the female stage in the laboratory stock but was not observed since this stock was not frequently examined during the period concerned.

## DISCUSSION

Two forms of head reconstitution occur in *S. grande* and in *S. tenuicauda:* In the one, a complete new head forms at the anterior end of the piece without the occurrence of resorption. The process of the development of a head at the cut end is doubtless more or less similar to that in other turbellarians. The cells at the anterior cut surface begin the development of a new individual as in ontogeny, the head differentiating first and organizing the anteroposterior development of new structures. The other form of head reconstitution, the process of resorption, is of considerable interest and importance; the cells at the anterior end of the piece do not differentiate as a head independently of posterior regions, but instead are so completely dominated by the head region at a posterior fission plane that resorption of the whole partial zooid occurs and the former posterior head becomes the new anterior head of the reconstituted piece. If a complete younger zooid, whose fission plane is less developed than that of the resorbing zooid, intervenes between the latter and the anterior partial zooid, it is also resorbed. The occurrence of resorption indicates that influence of a head may be exerted anteriorly as well as posteriorly, and in the abscence of a more highly developed head not only may inhibit development of a more anterior region but may bring about its physiological disintegration as an individual and its resorption. In such cases the development of the region concerned has taken place under the dominance of its own head region; but when this is removed, it is subjected to correlative action from the more posterior head region, i.e., an action opposite in direction to the original, and apparently under this reversal of the direction of the correlative conditions it can no longer persist. The influence of the posterior head must tend to obliterate and reverse the gradient of the region concerned. Resorption of anterior zooids or parts by a posterior zooid takes place only when the anterior zooid represents an earlier stage of development than the posterior or is headless. A more advanced head an-

terior to one less advanced is able to maintain itself. The persistence and development of the zooids in the normal chain apparently depend on the fact that the most advanced head region always constitutes the anterior end of the chain. If this head region is removed, the remainder of even the anterior zooid may be resorbed like any other headless zooid (Figs. 3–8).

This fact indicates that the head is the chief factor in the maintenance of a zooid against the influence of more posterior zooids. On the other hand, the fact that a posterior head region without the body of its zooid may resorb a complete zooid of earlier stage anterior to it (Child, 1903, Plate I, Figs. 19–26) shows that the head region is the chief factor in resorption. In short, the head is evidently the dominant region of the zooid, and its effectiveness increases as development progresses. If this is true, there can be little doubt that the dominance of the head region is transmissive in character and that its effectiveness and range depend in large part on the functional development of the nervous system. Data on length of zooids to appear in a later paper furnish further evidence in support of this conclusion.

Chains which were irradiated by the quartz mercury arc separated into single zooids; and in many of these, after more or less recovery, adventitious heads appeared. Such heads also appeared in old experimental cultures in which the worms also showed extreme derangement of normal zooid succession and length relations with reversal of the gradient of zooids indicated in some cases by a head at the posterior end of the zooid pointing in the opposite direction from the normal head (Figs. 37–47). Although it was not possible to control experimentally the production of such adventitious heads on the whole undivided chain of zooids, there would seem to be no essential difference between these heads and those which can be obtained experimentally by exposure to ultra-violet radiation. In both cases the gradient is probably obliterated and subsequently replaced by new polarities, which in some cases may be in the reverse direction of the original. The localization of the new heads is apparently determined fortuitously by several regions recovering at about the same time.

The fact that the female stage of sexual maturity of *S. grande*

shows no capacity for head reconstitution at any level posterior to the cephalic ganglia is interesting in view of the readiness with which pieces of the asexual chain reconstitute a head. That this loss of capacity includes uninjured tissue and is not merely a change in reaction to section is shown by the absence of fission planes and great length of body in the female individual. This situation indicates that with the development of femaleness the body of *Stenostomum* attains a higher degree of differentiation, and in this stage it resembles the rhabdocoels which do not undergo fission, as regards capacity of head reconstitution. So far as these have been examined, none of them is able to reconstitute a head at levels posterior to the cephalic ganglia. Only the Catenulidae, to which family *Stenostomum* belongs, and the Microstomidae, which also undergo fission and form chains, are able to reconstitute heads at any levels. E. H. Harper (1903) has noticed a decrease in the capacity for reconstitution with the appearance of sexuality in a microdrilous oligochaete. Evidently in the asexual stages of these forms the body cells have not attained their full differentiation.

*Stenostomum leucops* differs from *S. grande* and *S. tenuicauda* in the absence of resorption, in the low frequency of head-reconstitution, in the slower development of the head when it is reconstituted, and in the high frequency of development of new fission planes in the headless pieces. These differences suggest that in *S. leucops* the influence of more posterior on more anterior levels is less effective than in the other two species in bringing about resorption but more effective in inhibiting the development of heads in a large proportion of the cases. Apparently after the piece is cut and freed from the original dominance, a new head and fission plane usually begin to develop more rapidly at some level posterior to the cut surface than development of a head at the anterior cut end can occur; consequently, development of the anterior head is frequently inhibited. In other words, head development at a cut end, as compared with head development in uninjured tissue with formation of a fission plane, is less rapid in *S. leucops* than in the other two species, and consequently the head at the cut end is frequently inhibited; while in *S. grande* and *S. tenuicauda* the development of a head and fission

plane at more posterior levels is usually inhibited by the rapid development at the cut end.

But the development of a head and fission plane at some level posterior to the anterior cut end is not the only factor inhibiting head formation in *S. leucops*, for pieces often remain headless even when no fission plane develops in them. Nor can the inhibition of head formation in such cases be due entirely to stimuli originating from the injury and increased physiological activity at a posterior cut end for pieces which bear normal tails as well as those with a posterior cut end often remain headless. In general, pieces which remain headless without developing a fission plane are shorter than those which develop heads, i.e., head frequency apparently decreases to some extent with decreasing length of piece, whether or not a posterior cut end is present. Failure to reconstitute a head cannot be due to absence of capacity, for heads develop at fission planes at all levels. Apparently head formation is inhibited in some way.

It has been shown by many different lines of evidence that in *Planaria dorotocephala* the reconstituted head develops in spite of the rest of the piece (Child, 1914*a*, 1914*b*, 1916, 1920, 1921; Buchanan, 1922; Hinrichs, 1924), i.e., head formation represents an independent reaction of the cells concerned which is not determined by the rest of the piece but which may be inhibited by it. Doubtless head formation at the anterior cut end in *Stenostomum* is similar as regards its relations to other levels of the piece.

If we admit this, the facts as they stand indicate that in *S. leucops* the reaction of the cells at the cut end is usually inhibited by more posterior levels whether or not they include a head or a posterior cut end, but that this inhibition is not sufficient to bring about resorption in this species.

Summing up, as regards differences in head frequency of these three species: In *S. grande* and *S. tenuicauda* a head region at a level posterior to a less advanced head region or a cut end not only may inhibit head formation but may completely resorb the more anterior zooid or part of a zooid; but in the absence of a more posterior head, head formation at the cut end is not inhibited. In *S. leucops*, on the other hand, resorption does not occur, but head for-

mation may be inhibited, either by a more posterior head or apparently by other regions in the absence of such a head. At present nothing can be said concerning the nature of the factors determining these specific differences.

That the low head frequency in *S. leucops* can be modified and controlled to some extent is evident, however, both from the differences in head frequency in freshly collected and in laboratory material and from the increase in head frequency at low temperature. It is hoped that further investigation may provide a basis for more complete control and so throw further light on the factors concerned.

## SUMMARY

1. The reconstitution of *Stenostomum tenuicauda* and of *S. grande* in pieces containing one or more fission planes in the absence of the head of the chain is marked by the resorption of anterior partial zooids and of younger zooids by an older posterior head region. Pieces containing no fission planes at the time of section reconstitute a head at the anterior cut end in the majority of cases, and rarely form new fission planes while headless.

2. Resorption in *S. grande* and *S. tenuicauda* results from the influence of a posterior head region or regions, and occurs only when no head, or a head less advanced in development than the resorbing head, is present in the region resorbed.

3. *Stenostomum leucops* differs in reconstitutional behavior from *S. grande* and *S. tenuicauda* in the absence of resorption under any circumstances, in the low frequency of head reconstitution at the anterior cut end, in the slower development of the head when it is reconstituted, and in the high frequency of development in the piece of new fission planes in the absence of the head of the chain.

4. The low head frequency in pieces of *S. leucops* cannot be due to lack of capacity of the cells, for heads develop in connection with fission planes at all possible levels posterior to the primary head. Apparently, then, the low head frequency must be due to inhibition of head formation in some way.

5. The head frequency of *S. leucops* differs somewhat with different conditions and can be modified experimentally to some extent. Pieces from freshly collected animals show a head frequency

more than twice as high as laboratory cultures at room temperature, but laboratory cultures at low temperature give a head frequency almost as high as freshly collected animals. In general, long pieces in the absence of a new fission plane show a higher head frequency than shorter.

6. *Stenostomum leucops* lives and grows well in a wide range of different culture media, but no appreciable modification of head frequency in pieces was obtained with any of the media at room temperature.

7. Attempts to alter head frequency by means of KCN, ethyl urethane, chloral hydrate, chloretone, strychnin sulphate, and caffein were unsuccessful.

8. All the observations suggest that development of fission planes in headless pieces of *S. leucops* is determined by the removal of the dominant head region of the zooid, which decreases the range of dominance of the anterior end and permits physiological isolation and development of a head at some level of the piece. Apparently in *S. leucops* such a head develops more rapidly than the head at the cut end and constitutes a factor in the inhibition of development of the latter. In other words, in *S. leucops* a more posterior head does not resorb a more anterior less-developed head, as in *S. grande* and *S. tenuicauda;* but a more posterior head, and apparently also a more posterior region in the absence of a visible head, may inhibit head development at the anterior cut end. Further work is necessary to explain the nature of the factors determining the specific differences observed in reconstitutional behavior.

9. The demonstrated association of physiological dominance in *Stenostomum* with head regions and with the degree of development of such regions indicates that dominance is exerted by transmissive processes which depend on the functional development of the nervous system.

10. The production of adventitious heads by the use of ultraviolet radiation shows the capacity of all levels of the chain of *S. leucops* to produce heads, in this case following obliteration of the original gradient and subsequent recovery of several regions at about the same time. This same capacity is evident from the development of fission planes.

11. The female stage of sexual maturity of *S. grande* shows no capacity to reconstitute a head at any level posterior to the cephalic ganglia. This loss of capacity included uninjured tissue as well as cut surfaces, for the female individual apparently cannot form fission planes.

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# THE CORPUS LUTEUM HORMONE. I. EXPERIMENTAL RELAXATION OF THE PELVIC LIGAMENTS OF THE GUINEA PIG[1]

(Two plates)

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## INTRODUCTION

THE extraordinary separation of the pelvic bones of the guinea pig during pregnancy has been observed by many investigators. Perhaps the first to call attention to this was Duncan (1854); while later it was also mentioned by Owen (1866), Flower (1885), Stirling (1902), and Bland-Sutton (1911). Whitley (1911) added the important observation that the separation of the pubic bones was a gradual process. These discussions were concerned chiefly with the fact that separation occurs, and it was not until the careful studies of Todd (1923) and Kawata (1925) that the anatomical and histological aspects of this phenomenon were known.

## STATEMENT OF THE PROBLEM

Chapman (1919), in his work on the Geomyidae, found that some of the females had a complete symphysis pubis while in others not only was the symphysis absent but the pubic bones were very widely separated. In the male Geomyidae the pubic bones were always united at the pubic symphysis by a well-established articulation. The writer (Hisaw, 1925) confirmed Chapman's observations but noticed that those females having the complete symphysis were virgins and that the pubic bones were gradually resorbed at the approach of sexual maturity. These facts seemed to indicate that the disappearance of the pubic symphysis in these animals was due to some physiological factor associated with reproduction. Experiments conducted to test this assumption demonstrated that this peculiar change in the pubic bones of the pocket gopher acted as a

[1] This work has been assisted by grants from the Committee for Research on Problems of Sex of the National Research Council.

secondary sexual character and was governed by the ovaries. It was found that males, which ordinarily retain the pubic bones throughout life, could be made to lose them if they were castrated and given ovarian extracts, and that young castrated females, into which testicular grafts were repeatedly made, retained their pubic symphyses. That is, the loss of the pubic symphysis of feminized males is associated with the development of the female secondary sexual characters, while the retention of the symphysis by masculinized females accompanies the growth of sexual characteristics peculiar to the male. These facts made it seem probable that resorption of the pubic bones of Geomyidae may have a general bearing on the metamorphosis of all Eutherian pelves and to ligamentous relaxation found in certain mammalian pelves during pregnancy. An animal well adapted to investigations of this kind is the common guinea pig, as it demonstrates very marked ligamentous relaxation during pregnancy and, according to Kawata, shows pronounced resorption of the pubic bones. Flower describes the symphysis of the guinea pig as being ligamentous and adds that the bones are widely separated at parturition. Chapman, in his discussion of the guinea-pig symphysis, speaks of it as a "generalized state" and compares it to that of beavers—"where the caudal vertebrae are free and a well-developed symphysis is present." Todd called attention to the apparent contradiction of these statements and suggested that "Flower's description refers to the multiparous guinea pig, whereas the statement by Chapman is correct only for the virginal animal." It is quite probable that these investigators were not familiar with or overlooked the possibility of a sex dimorphism of the guinea-pig symphysis. Histological examinations show, as will be pointed out in this paper, that the symphysis pubis of the virgin female does remain ligamentous, while that of the male is firmly united by cartilage. The investigations reported in this paper are, however, concerned chiefly with a physiological explanation of the anatomical changes in the pelvis of the guinea pig during pregnancy.

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### EFFECTS OF DRIED OVARIAN MATERIAL AND THE FOLLICULAR HORMONE

The changes produced in the pubic bones of the pocket gopher were induced chiefly through the use of hot saline extracts of dried ovarian substance, and in the initial experiments on the guinea pig this same substance was employed. A series of normal males and females of various ages, castrated young males, and castrated mature males were given three to four 5 cc. doses intraperitoneally per week for a period, in many cases, of 6 months. A total of some fifty animals was used, and not a single instance of relaxation of the pubic symphysis was noticed. Some of the castrated males, however, showed a change in the pubic symphysis in that the pubic bones were not as firmly united as in the normal. It is very probable that the pubic symphyses of these males were feminized, and these cases will be treated later in a part of this paper which deals with the relaxation of the male pelvis.

The experiments with the dried ovarian material were made before Allen and Doisy (1923) discovered their method of extracting the follicular hormone, so it was of interest to find the importance of this substance with regard to the problem in hand. These investigators furnished me with an oil preparation obtainable from human placentae which contained 72 rat units per cubic centimeter. With this substance it was possible to administer large doses of the follicular hormone and keep both castrated and normal female guinea pigs in full oestrum for extended periods. These experiments were also repeated with follicular hormone which the writer prepared and with samples of the same hormone furnished by E. A. Squibb and Sons, with entirely negative results as far as relaxation of the pelvic ligaments was concerned.

### BLOOD SERUM OF PREGNANT RABBITS

The negative results reported above seem to prove quite conclusively that relaxation of the pelvic ligaments of a guinea pig must have a different physiological explanation from that of the resorption of the pubic bones of a pocket gopher. After searching for another explanation, it was concluded that if this reaction depends on a hormone, the substance must be carried in the blood; and that as relaxation of the pelvic ligaments takes place normally only during

pregnancy, the blood of such animals would be a likely source. This assumption was tested by injecting blood serum of pregnant rabbits into virgin female pigs.

It was found that, when 2 cc. of blood serum from pregnant rabbits were injected intraperitoneally into virgin guinea pigs, the great majority of the animals showed very noticeable relaxation, which could be easily diagnosed by palpation. This relaxation could be demonstrated, in many cases, 8 hours after injection; but it usually reached its maximum in 12–18 hours, the pelvis returning to its normal condition within 2 or 3 days. The ligamentous relaxation obtained ranged from conditions which were very pronounced, where the two halves of the pelvis could be moved freely, to those in which movement only slightly more than normal could be detected, while a few failed to respond. In the most favorable cases the pubic bones could be easily separated 3–4 mm. (Plate I, Fig. 1), and presented a condition which seemed to be identical with that of early pregnancy.

The importance of explaining why some of the animals relaxed so very much better than others and why a few failed to respond is obvious. The most conspicuous variable factor, which was not controlled in the experiment, was the stage of the oestrous cycle when the pregnant serum was injected. A study of this point demonstrated conclusively that the oestrous condition is of great importance in the response of the pelvic ligaments to the serum treatment. When virgin guinea pigs were injected with 2 cc. of pregnant rabbit serum during oestrum and early post-oestrum, pronounced relaxation always followed. Out of several hundred guinea pigs that were injected, only one was found that could not be relaxed at this time of the oestral cycle. Relaxation could be produced, but in decreasing amounts, up until the sixth and seventh day after oestrum, at which time the reactions became erratic; from the eighth to the eleventh day little or no relaxation occurred; on the twelfth and the thirteenth day some response may be had; and from this time until the following oestrum the effects of the pregnant serum gradually became more pronounced.

The most striking thing about these results is the correlation between the time that relaxation of the pelvic ligaments can be

induced and the history of follicular development. That is, the greatest relaxation was obtained at or near full oestrum, which is the point in the oestrous cycle when the animal is under the maximum

TABLE I

SHOWING CHANGES IN THE PELVIC LIGAMENTS OF VIRGIN GUINEA PIGS INDUCED BY THE INJECTION OF 1 CC. OF PREGNANT RABBIT SERUM INTRAPERITONEALLY ON DIFFERENT DAYS OF THE OESTROUS CYCLE. EACH ANIMAL WAS TESTED ONLY ONCE DURING A SINGLE CYCLE. THE FIRST THREE DAYS OF THE OESTROUS CYCLE WERE NOT INCLUDED IN THE TABLE AS RELAXATION ALMOST INVARIABLY FOLLOWS SERUM TREATMENT DURING THAT PERIOD.

(Day of injection is indicated by "x")

| ANIMALS | DAYS OF NORMAL OESTROUS CYCLE | | | | | | | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | |
| C388.2 | x | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | Very relaxed |
| C388.2 | ... | x | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | Very relaxed |
| 472 | ... | x | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | Not relaxed |
| 445 | ... | ... | x | ... | ... | ... | ... | ... | ... | ... | ... | ... | Not relaxed |
| 456 | ... | ... | x | ... | ... | ... | ... | ... | ... | ... | ... | ... | Not relaxed |
| 421 | ... | ... | ... | x | ... | ... | ... | ... | ... | ... | ... | ... | Not relaxed |
| 453 | ... | ... | ... | x | ... | ... | ... | ... | ... | ... | ... | ... | Slightly relaxed |
| 456 | ... | ... | ... | ... | x | ... | ... | ... | ... | ... | ... | ... | Not relaxed |
| 453 | ... | ... | ... | ... | x | ... | ... | ... | ... | ... | ... | ... | Slightly relaxed |
| 421 | ... | ... | ... | ... | x | ... | ... | ... | ... | ... | ... | ... | Not relaxed |
| 439 | ... | ... | ... | ... | ... | x | ... | ... | ... | ... | ... | ... | Not relaxed |
| 457 | ... | ... | ... | ... | ... | x | ... | ... | ... | ... | ... | ... | Not relaxed |
| 457 | ... | ... | ... | ... | ... | ... | x | ... | ... | ... | ... | ... | Not relaxed |
| 21 | ... | ... | ... | ... | ... | ... | x | ... | ... | ... | ... | ... | Slightly relaxed |
| 21 | ... | ... | ... | ... | ... | ... | ... | x | ... | ... | ... | ... | Fairly relaxed |
| 468 | ... | ... | ... | ... | ... | ... | ... | x | ... | ... | ... | ... | Not relaxed |
| 469 | ... | ... | ... | ... | ... | ... | ... | ... | x | ... | ... | ... | Not relaxed |
| 450 | ... | ... | ... | ... | ... | ... | ... | ... | x | ... | ... | ... | Not relaxed |
| 453 | ... | ... | ... | ... | ... | ... | ... | ... | x | ... | ... | ... | Slightly relaxed |
| 421 | ... | ... | ... | ... | ... | ... | ... | ... | ... | x | ... | ... | Slightly relaxed |
| 472 | ... | ... | ... | ... | ... | ... | ... | ... | ... | x | ... | ... | Slightly relaxed |
| 472 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | x | ... | Slightly relaxed |
| 494 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | x | ... | Slightly relaxed |
| 450 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | x | Not relaxed |
| 456 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | x | Slightly relaxed |

influence of the follicular hormone. The least relaxation and negative responses occurred at the di-oestrum, when follicular secretion is at its minimum.

## RELAXATION OF THE PELVIC LIGAMENTS OF CASTRATED VIRGIN GUINEA PIGS

The results just reported raised the point as to whether or not the follicular homone was essential for the relaxation reaction. This

## PLATE I

FIG. 1.—Transverse section of fully relaxed symphysis pubis of a virgin guinea pig. This condition was produced by a single intraperitoneal injection of 2 cc. blood serum of a pregnant rabbit. About half of the pubic ligament is shown, and a section of one pubic bone may be seen at the left.

FIG. 2.—Transverse section of the symphysis pubis of a mature male guinea pig, showing a cartilaginous union without an interpubic cleft.

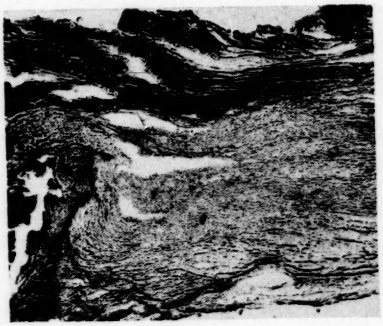
FIG. 3.—Transverse section of the symphysis pubis of an old male guinea pig. The cartilaginous symphysis has been in large part replaced by bone.

FIG. 4.—Transverse section of the symphysis pubis of a mature, virgin, female guinea pig, showing dorsal and ventral ligaments and interpubic cleft.

FIG. 5.—Transverse section of the symphysis pubis of a mature female guinea pig which was castrated soon after birth. The symphysis did not develop into the female type but remained like that of males as shown in Figure 2.

FIG. 9.—Transverse section of the symphysis pubis of a young female, showing the beginning of an interpubic cleft.

PLATE I



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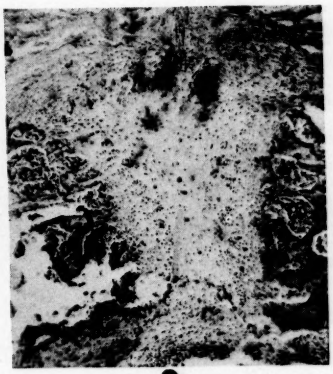

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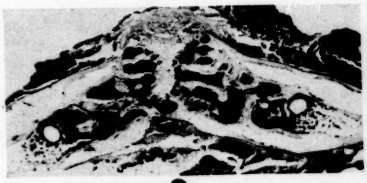

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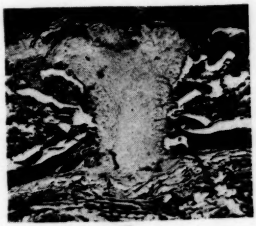

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was determined by experiments on castrated, virgin, female guinea pigs. Ten animals were used in the first test, and each received 2 cc. of pregnant rabbit serum. The pelvic ligaments of eight of this number did not relax, while two showed pronounced movement at the symphysis pubis and were found later to be incompletely castrated. This experiment was repeated several times, and it has been conclusively shown that a guinea pig which is completely ovariotomized is not relaxed by the serum treatment. However, when these castrated animals were brought into artificial oestrum, by the injection of follicular hormone, and then given pregnant rabbit serum, the pubic ligaments relaxed. It was also found that fair relaxation could occasionally be produced by injecting about 1 rat unit of follicular hormone, which was not enough to produce oestrum, followed on the next day by 2 cc. of pregnant rabbit serum. When both the serum and the follicular hormone were injected at the same time, relaxation did not follow on the first injection; but when this treatment was continued long enough for the follicular hormone to have its effect, some relaxation was produced.

These results demonstrate that two substances are involved in pelvic relaxation, namely, the follicular hormone and a second substance in pregnant blood, and that neither one is capable of producing the reaction alone. In this case it seems that the animal must first be put in the proper physiological condition by the follicular hormone before the pelvic ligaments are able to respond to the substance present in pregnant rabbit serum. It is also important to note that relaxation cannot be produced in castrated females by first giving blood serum and then following with follicular hormone, which shows that it is a "one two" reaction which is not reversible.

### RELAXATION OF THE PELVIC LIGAMENTS OF MALE GUINEA PIGS

When normal, castrated male guinea pigs are injected with serum of pregnant rabbits, relaxation is not produced, even though they have received follicular hormone. This is due to an anatomical difference between the male and the female pubic symphysis. In the matured male, the pubic bones are united by cartilage, especially at their anterior ends (Plate I, Fig. 2); while in old males, this cartilaginous structure is replaced by bone to a greater extent (Plate I,

Fig. 3), thus causing a very rigid union. The symphysis pubis of adult females remains ligamentous throughout life (Plate I, Fig. 4), or at least this is true for virgin animals over three years old. That the ovary plays an important part in the metamorphosis of the cartilaginous symphysis of sexually immature guinea pigs to the ligamentous condition can be demonstrated by castrating very young females. The pubic symphysis of these animals retains the cartilaginous condition characteristic of males (Plate I, Fig. 5) and cannot be relaxed by the procedure just described for females that were castrated after sexual maturity. This sex dimorphism is very suggestive of the condition present in pocket gophers (Hisaw, 1925). It was found that in these animals the pubic bones of both males and females became completely ossified to form a rigid symphysis pubis, but that these bones in the young females are extensively resorbed with the approach of the first oestrum, while those of males are retained throughout life. It was further established that the resorption of the pubic bones was due, at least in part, to the influence of the ovary, and that a similar condition could be brought about in castrated males feminized by means of injected ovarian extracts. This work made it seem probable that the structure of the symphysis pubis of male guinea pigs could be modified by feminization. Fourteen castrated male guinea pigs were injected with follicular hormone daily for from 16 to 71 days, receiving a total of from 19 to 61 rat units. This caused marked mammary growth, and in three cases some change in the pubic symphysis was produced. This method required considerable follicular hormone, which had to be injected for many days, and the results were not as pronounced as desired, so it was abandoned in favor of feminization by ovarian grafts. When the grafts were successful, the mammary glands grew very large, several lactated, and a few animals showed marked changes in the symphysis pubis. Those showing symphyseal modifications gave unquestionable responses to the injection of relaxation hormone but never to the great degree characteristic of females in oestrum.

Histological examination of the pelves of feminized males revealed that the cartilage of the pubic symphysis had not been entirely replaced by connective tissue (Plate II, Figs. 6 and 7). It seems that cartilaginous resorption begins at the posterior end of the

symphysis and progresses anteriorly. The amount of relaxation depends upon the degree of feminization of the pubic symphysis. The time required to produce modifications of a cartilaginous structure, such as the male symphysis pubis, seems to be considerably longer than that for such structures as the mammary glands. It is possible to have mammary growth sufficient for lactation before the pubic symphasis is feminized enough to give a satisfactory relaxation. The fact that there is a pronounced sex dimorphism in the pelvic girdle of the guinea pig and the pocket gopher, and that the symphysis pubis in both cases can be modified by ovarian influences, seems to indicate the possibility that this process may be of importance in the metamorphosis of the pelves of many Eutherian mammals. It should be mentioned, however, that this cannot be made as a sweeping statement, for at least the moles seem to be an exception. The embryos of *Scalopus aquaticus machrinus* (Hisaw and Zilley, 1927) and *Talpa europaea* (Leche, 1880) have complete cartilaginous pubic arches, but these do not become ossified and are lost in both males and females. Here it seems that an endocrine, at least from the reproductive system, is not involved.

### SOURCE OF THE RELAXATIVE SUBSTANCE IN THE BLOOD OF PREGNANT RABBITS

It was assumed that the blood of pregnant rabbits acted only as a carrier of the relaxative substance and that it was a hormone secreted by some ductless gland. The first indication that this hypothesis was correct came from a study of the time of appearance and disappearance of the substance in the blood during pregnancy. It was found that the substance was first present on the seventh to eighth day in sufficient amount so that 2 cc. of the serum would produce noticeable relaxation of the pelvic ligaments of the guinea pig, and that the blood lost this property within 12–18 hours after parturition. This very clearly demonstrated that a source for the formation of the hormone was lacking after the loss of the fetuses and their associated membranes. Extracts of the maternal and fetal parts of the rabbit placenta were tested for the presence of the hormone, and constant excellent positive reactions resulted. Similar tests were also made for the amniotic liquor, fetal blood, and the fetus. The relaxation obtained from these sources was erratic, the

## PLATE II

FIG. 6.—Transverse section of the symphysis pubis of a castrated male guinea pig which has been feminized by ovarian grafts. A part of each pubic bone is at the right and left. The cartilage of the symphysis is not completely replaced but is separated from the pubic bones by connective tissue.

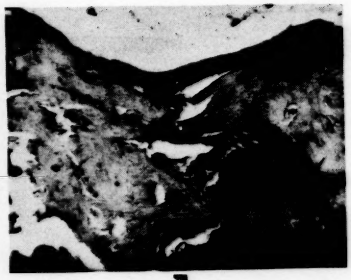
FIG. 7.—Transverse section of the symphysis pubis of a castrated male guinea pig which has been completely feminized by ovarian grafts and relaxed. Part of the right pubic bone is at the edge of the picture. The symphysis is completely ligamentous as shown, but parts of the symphyseal cartilage could be bound in the ligament at the anterior end.

FIG. 8.—Transverse section of the pubic symphysis of a virgin female guinea pig which was relaxed by a single subcutaneous injection of 0.1 cc. of corpus luteum extract which was equal to 1 gm. of fresh sow corpus luteum. The relaxation shown is not as pronounced as that produced by a larger dose.

FIG. 10.—Frontal section of the pubic symphysis of a mature male guinea pig, showing relation of the cartilaginous symphysis to the pubic bones.

PLATE II

6



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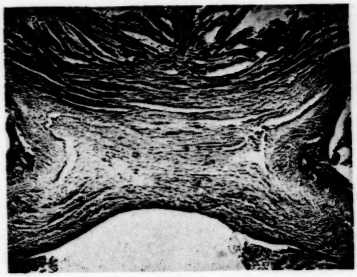

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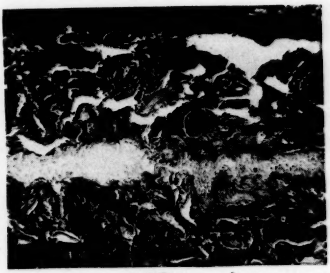

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fetal blood being more constant than the other two, so it was concluded that the hormone was not formed in the body of the embryo and that the positive results were perhaps due to close association of these structures with the placenta.

Additional information was gained through the study of the blood of rabbits which were castrated during pregnancy. The great majority of these animals aborted within a day or two; but two retained their young, one for 5 days and the other for 8 days. In all these animals it was found that the blood retained its relaxative ability until the fetuses were discharged. The same experiments were performed on guinea pigs, and it was found, in the few cases where abortions did not follow, that the absence of the ovaries did not prevent further relaxation of the pelvic ligaments as pregnancy progressed. The relatively brief period during which the hormone is present in the blood after parturition indicates that it is rapidly eliminated from the body, and the fact that it is present in what seems to be a normal amount, as long as the fetuses are retained, points toward the placenta as at least one source for its formation.

The positive reaction after the injection of the rabbit serum is so constant that it can be used as an excellent method for diagnosing pregnancy. This has been tried by obtaining samples of blood from a strange rabbit colony where detailed breeding-records were kept, and testing their reactions on virgin guinea pigs in full oestrum. Those samples which relaxed the pubic ligaments signified pregnancy of the animals from which they were taken. If one is able to read the vaginal smears of the guinea pig, the efficiency of this method for the rabbit is almost 100 per cent.

### PRESENCE OF THE RELAXATIVE HORMONE IN MAMMALS OTHER THAN THE RABBIT

A study of blood from pregnant animals revealed wide variations in content of the relaxative hormone. Blood from pregnant sows, dogs, cats, and mares gave positive results, while that of cows and women was entirely negative. The quantitative criterion used in this work was the dosage of serum required to produce relaxation when injected intraperitoneally into a guinea pig. The rabbit was found to rank first, requiring only 0.25 cc. of serum to produce noticeable changes. This is very much better than guinea-pig blood, which was

found to be poor in the hormone, from 6 cc. to 10 cc. given in 2 cc. doses for 3–4 days being necessary to cause even fair relaxation. It took nine 2 cc. doses on as many consecutive days to produce a fair relaxation when dog serum was used. The blood of cats and sows

TABLE II

SHOWING CHANGES IN THE PELVIC LIGAMENTS OF VIRGIN GUINEA PIGS INDUCED BY A SINGLE SUBCUTANEOUS INJECTION OF 0.1 CC. OF CORPUS LUTEUM EXTRACT GIVEN ON DIFFERENT DAYS OF THE OESTROUS CYCLE. THIS DOSAGE WAS EQUAL TO 1 GM. OF FRESH TISSUE AND WAS GIVEN ONLY ONCE DURING A SINGLE OESTROUS CYCLE

(Day of injection is indicated by "x")

| ANIMALS | DAYS OF NORMAL OESTROUS CYCLE | | | | | | | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | |
| 468 | x | | | | | | | | | | | | Relaxed |
| 469 | x | | | | | | | | | | | | Relaxed |
| 472 | | x | | | | | | | | | | | Relaxed |
| 439 | | x | | | | | | | | | | | Slightly relaxed |
| 445 | | | x | | | | | | | | | | Slightly relaxed |
| 422 | | | x | | | | | | | | | | Not relaxed |
| 21 | | | | x | | | | | | | | | Not relaxed |
| 456 | | | | x | | | | | | | | | Not relaxed |
| 472 | | | | x | | | | | | | | | Not relaxed |
| 445 | | | | | x | | | | | | | | Not relaxed |
| 454 | | | | | x | | | | | | | | Not relaxed |
| 21 | | | | | | x | | | | | | | Not relaxed |
| 457 | | | | | | x | | | | | | | Not relaxed |
| 453 | | | | | | | x | | | | | | Slightly relaxed |
| 439 | | | | | | | x | | | | | | Not relaxed |
| 468 | | | | | | | x | | | | | | Not relaxed |
| 454 | | | | | | | | x | | | | | Not relaxed |
| C388.2 | | | | | | | | x | | | | | Slightly relaxed |
| 422 | | | | | | | | | x | | | | Not relaxed |
| C388.2 | | | | | | | | | x | | | | Fairly relaxed |
| 468 | | | | | | | | | | x | | | Not relaxed |
| C388.2 | | | | | | | | | | x | | | Fairly relaxed |
| 456 | | | | | | | | | | | x | | Fairly relaxed |
| 457 | | | | | | | | | | | x | | Fairly relaxed |
| 494 | | | | | | | | | | | | x | Fairly relaxed |
| 453 | | | | | | | | | | | | x | Relaxed |

was sufficiently potent so that 2 cc. were ample to produce good positive results, while that of the mare was poor.

Just why the blood of cows and women gave negative results is not clear. The human blood was obtained from three women during the seventh, eighth, and ninth months of pregnancy; and after injecting the serum into guinea pigs for several days, no change in the

pelvic ligaments could be detected. It was first thought that these results were due to low concentration of the hormone, but it was not possible to demonstrate its presence even by injecting extracts of 40 cc. samples in a single dose. At a later time extracts of the human and the cow placenta were made, but they had no noticeable effect on the guinea-pig's pelvic ligaments.

A probable explanation of these results may be found in the balance which perhaps exists between the follicular hormone and the relaxative substance. It is a well-recognized fact that cow and human placentae and human blood during pregnancy contain a very high percentage of follicular hormone, and it may be that this is present in sufficient amount to smother effects of the relaxative hormone. This point can be settled only when a technique for fractionating these substances is known.

One of the things noticeable on analysis of these data is that the relaxative hormone was found in animals having very different types of placentae. It was thought quite possible that this hormone, like the oestrous hormone of the Graafian follicles, was also formed in other tissues than the placenta. This led to a study of the sow corpus luteum and the discovery that this gland was a very excellent source for the substance. It yet remained, however, to prove that the active agent here was the same as that found in pregnant serum, so all the work on relaxation of the pelvic ligaments was repeated with corpus luteum extracts (Table II). This study established the fact that the corpus luteum hormone acted on the ligaments of the pelvic girdle under the same physiological conditions as those found for the relaxative substance in pregnant serum. Not only is this true, but the chemical properties established for the hormone in pregnant blood are the same as those of the hormone extracted from corpora lutea and placentae.

### HISTOLOGICAL NATURE OF THE RELAXATION PROCESS

The pubic bones of a sexually mature, virgin, female guinea pig are held firmly together by connective tissue (Plate I, Fig. 4). This connective tissue may be divided into three regions: thick dorsal and ventral bands which extend across the symphysis and attach on the dorsal and ventral surfaces, and a third mass in the symphysis be-

tween the opposing surfaces of the pubic bones. In the early stages of relaxation, which begins within 8–12 hours when induced by corpus luteum hormone, multiplication of connective tissue does not seem to occur. The relaxation at this time is a result of the migration of the dorsal and ventral connective tissue bands into the symphysis, thus allowing the pubic bones to move apart (Plate II, Fig. 8). This shifting of the ligaments seems to be correlated with the amount of corpus luteum hormone injected, as different degrees of relaxation in a general sense can be produced by giving small or large doses. The relaxed condition may remain for only a day or for several days, depending upon the amount of change produced, after which the bones and ligaments return to their former position. When a guinea pig in full oestrum is given corpus luteum daily, the reaction cannot be augmented and sustained indefinitely. After about the sixth or the seventh day there is a slight response after each injection, followed by a restoration of almost the normal condition within the next 24 hours. In order to produce prolonged relaxation associated with connective tissue growth of the pelvic ligaments, characteristic of late pregnancy, the animal must also be given follicular hormone during the time of corpus luteum treatment.

The exact cytological activity of the symphyseal tissue during relaxation has not been thoroughly studied, but it should be noted that the characteristics of the pubic ligaments at their attachments to the pubic bones differ from the ordinary in that many nuclei are present, signifying undifferentiated connective tissue cells and the possibility of rapid cellular growth. When relaxation is carried to a point where it is associated with considerable cellular growth of the connective tissue in the symphysis, the normal virginal condition is not restored. This work also confirms the observations of Todd (1923) and Kawata (1925) on the normal guinea pig during pregnancy, in that they found the virginal condition of the symphysis was not restored after pregnancy. In these cases there is a great multiplication of connective tissue which is not entirely resorbed after parturition. With each succeeding pregnancy there is an accumulation of tissue in the symphysis, so that it is not only possible to distinguish a virgin from a multipara but it is also possible, in a general way, to tell whether a non-pregnant female has delivered one litter

or several by determining the separation of the pubic bones. This can be diagnosed very easily by placing a finger on the symphysis pubis and alternately pushing up and down on the right and left ischia.

### SOME PROPERTIES OF THE CORPUS LUTEUM HORMONE

Some of the general properties of the corpus luteum hormone, as used in these experiments, are mentioned here with the conviction that some idea of the character of the substance should be given at this time, but a detailed description of methods of preparation and chemical characteristics are being reserved for a later paper. The hormone is soluble in acid solutions and may be extracted through their use. It may also be extracted from blood serum by neutral alcohol but not to advantage. It is thermolabile and is destroyed by drying. It dialyzes very slowly or not at all, and does not pass through an ultrafilter which holds back congo red or haemoglobin. When kept in blood serum or sterile aqueous solutions, it retains its potency at least for several weeks. The hormone can be salted out of blood serum or tissue juices where it is associated with the pseudoglobulin fractions. It cannot be extracted with ether, acetone, petroleum ether, or chloroform. Fair relaxation can be produced by giving large doses, 10–20 cc. by mouth.

### DISCUSSION

A question that naturally arises, in the light of the facts presented, is that concerning the evidence for believing the substance responsible for relaxation of the pelvic ligaments is the corpus luteum hormone. One reason for this is based on a study of the properties of the relaxative substance. No matter in what tissue it was found it could be extracted by the same chemical procedures, had the same properties, and acted under the same physiological conditions. If tissues or extracts containing the substance were dried, or exposed to temperatures of about 60° C. for 24 hours, it was always rendered inactive. No extracts studied were able to produce relaxation alone, and in all cases the animal had to be either under or recovering from the effects of the follicular hormone before the potent extracts could act.

Perhaps the most convincing evidence that the relaxative sub-

stance is a corpus luteum hormone is the ability of the substance to produce physiological reactions ordinarily attributed to the corpus luteum, such as the inhibition of oestrum and the production of placentomata. Through the use of these extracts oestrum of the rat can be inhibited for several cycles; and when injections are discontinued, oestrum usually follows within 4 or 5 days. Examinations of the vaginas of these animals revealed the histological pictures described by Long and Evans (1922) for the rat during normal pregnancy and during pseudo-pregnancy induced by stimulation of the cervix. The only difference between the condition produced by extracts and that ascribed to prolonged activity of normal corpora lutea is one of degree of cellular activity. The vaginal mucosa of rats receiving highly potent corpus luteum extracts reaches a greater height and is more extensively vacuolated than that of pregnancy or pseudo-pregnancy. One striking contrast between the inhibition of oestrum and relaxation of the pelvic ligaments is the amount of hormone required. The relaxation reaction seems to be rather delicate, requiring only a single dose of 0.1 cc. of the best extracts, which is equal to 1 gm. of tissue; while 1.5 cc. divided into several small doses and given at frequent intervals is required each day to inhibit oestrum in a rat. That is, it is necessary to inject fifteen times as much daily to inhibit oestrum in a rat as it does to relax a guinea pig which is in full oestrum. This no doubt suggests the advantage of the relaxation reaction as a test for potency of extracts over the usual procedure of inhibiting oestrum. This perhaps is sufficient for the present discussion, as the data on standardization and inhibition of oestrum will form the body of another paper in this series of studies.

The work of Loeb, Long and Evans, and others has clearly demonstrated the importance of the corpus luteum in the production of placentomata. They did not, however, emphasize the importance of the follicular hormone in this reaction and, it is thought, in some cases rather overemphasized the importance of the corpus luteum. Weichert (1928), who is working with the author on this phase of the problem and who will publish his results soon, has been able to produce placentomata in castrated rats and guinea pigs through the use of extracts. Here, again, it was demonstrated that the same

physiological conditions are required for decidual reaction as those described for relaxation, namely, the animals must first be put under the influence of the follicular hormone before the corpus luteum hormone can produce the growth of placentomata. Brouha (1928), in his work on the production of placentomata in rats, in which the life of the corpora lutea was prolonged by the injection of extracts of the anterior lobe of the hypophysis, demonstrated that the reaction could not be obtained beyond the tenth day after oestrum even while apparently functional corpora lutea were yet present. A probable explanation of this time limit may be that the effects produced by the follicular hormone have passed and the uterus is no longer physiologically able to respond to the corpus luteum hormone. These results seem to have a direct bearing on the opinion expressed by Novak (1928), who, in speaking of the therapeutic applications of ovarian extracts to human patients, says, ". . . . that injections of the corpus luteum in the human being, aside from uncertainties in the preparations used, might be expected to have little effect unless the endometrium was first prepared by injections of follicle hormone as in life."

The value of the relaxation reaction in diagnosing pregnancy in rabbits has been mentioned, but its general application to other animals is not known. It seems very probable that it would be equally efficient for those species where the corpus luteum hormone is abundant in the blood during pregnancy. One of the greatest disappointments in this study was the absolute failure of attempts to demonstrate the presence of the corpus luteum hormone in the blood of pregnant women. It should be mentioned, however, that the samples used were from women during the seventh, eighth, and ninth months. Frank and Goldberger (1928) have found that human blood during these months contains a very high concentration of follicular hormone, while during the early months it is not present in such great amounts. The idea that the corpus luteum hormone is lacking does not seem logical, and a more plausible explanation would be that it is physiologically overshadowed by the effects of the follicular hormone. Blood of early pregnancy would perhaps be favorable for this test. The same explanation may also apply equally well to the cow, where it was also not possible, at least by the technique used,

to demonstrate the presence of the corpus luteum hormone. Here, again, the opinion of Novak that the probable physiological importance of the corpus luteum and the follicular hormone of lower animals may differ from that of the human being seems well substantiated. The data collected in this paper demonstrate quite clearly that the corpus luteum hormone present in the blood, fetal membranes, or corpora lutea themselves varies greatly during pregnancy, from species to species, even between rather closely related forms as the rabbit and guinea pig.

A question that no doubt comes to the minds of those who read this paper is: If the relaxation of the pelvic ligaments produced by the corpus luteum hormone occurs only while the animal is under the influence of the follicular hormone, or shortly after, why is it that relaxation does not occur during the oestrous cycle? Out of the many animals examined, an occasional one was found which experienced very slight relaxation, usually during the early stages of oestrum. It should be emphasized that these cases were few and that ordinarily no change could be detected during the cycle. These instances are perhaps caused by persistent corpora lutea which continue to secrete after the follicles have reached a considerable size. It does not seem illogical to think that under unusual conditions the time relations of these two hormones may be brought closer together thus producing relaxation.

A question of interest, and one on which this study has thrown some light, is whether or not the placenta acts as a secretory organ for the corpus luteum and follicular hormones or is only a temporary storehouse for them after their formation by the ovary. The report here and that by Herrick (1928) show that ovariotomy does not stop the relaxation process in pregnant guinea pigs even though they are castrated before the middle of pregnancy. Although most of these animals aborted, a few went to term, gave birth to normal young, and lactated. It seems to be a well-established fact that the follicular hormone is responsible for mammary growth; while unpublished results of work in this laboratory indicate that the corpus luteum hormone, as extracted by us, is not capable of doing this. The fact that both mammary growth and relaxation occur in castrated pregnant guinea pigs demonstrated that both hormones are

present. Also, in castrated pregnant rabbits the corpus luteum content of the blood shows no appreciable decrease until after the loss of the placenta. These findings lead one to believe that at least the corpus luteum hormone, which is utilized rapidly in the body, is not stored but is secreted by the placenta.

Another question which cannot be answered from the data at hand but should be mentioned is: Why does abortion usually follow ovariotomy if the corpus luteum hormone is secreted by the placenta? It should be pointed out first that those few instances, in which abortion did not shortly follow ovariotomy, were confined entirely to animals operated on during advanced pregnancy. This has been found by several investigators to be true for our common laboratory rodents. Thus it seems that the corpus luteum is of the greatest importance during early pregnancy. It has also been found, though instances are not numerous, that abortion is less likely when the ovaries are removed at two operations separated by an interval of several days. This suggests the possibility of a compensatory hypertrophy of some source for the hormone to replace the loss of an ovary. Just what organ is involved is not known, but it may be the placenta.

## CONCLUSIONS

1. The relaxation of the pubic ligaments of the female guinea pig is under hormonal control and depends upon the co-operation of secretions from the Graafian follicles and the corpora lutea. The animal must be under the influence of the follicular hormone before the corpus luteum hormone can produce relaxation.

2. The pelvic ligaments of virgin female guinea pigs can be relaxed by the injection of the corpus luteum hormone during pro-oestrum, oestrum, and for the first 5 or 6 days of post-oestrum. Little or no relaxation follows this treatment during di-oestrum.

3. Castrated female guinea pigs can be relaxed only when they are first injected with follicular hormone and then given corpus luteum hormone.

4. It is not possible to relax the pubic symphyses of either normal or castrated male guinea pigs. This is due to a sex dimorphism of the symphyses, but the male condition can be changed to that of the female by feminization and relaxation then produced.

5. The corpus luteum hormone is found in the blood of the pregnant rabbit, guinea pig, sow, cat, dog, and mare. It was not found in the blood of pregnant women or cows. It may also be extracted from the maternal and fetal parts of the rabbit placenta but was not found in either human or cow placentae.

6. The corpus luteum hormone is soluble in acid solutions and can be extracted from tissues through their use. It may also be salted out of blood serum, where it is found associated with the pseudo-globulins. It is thermolabile and is killed by drying at room temperature. The hormone is not easily injured by acids but is destroyed by strong bases. It dialyzes slowly or not at all through collodion bags which hold back congo red and haemoglobin.

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# CONTROL OF SEX IN CLADOCERA. I. CROWDING THE MOTHERS AS A MEANS OF CONTROLLING MALE PRODUCTION[1]

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IT IS well known that Cladocera are usually parthenogenetic, producing only female young, and that for variable periods of time and numbers of generations the entire population of a pond may be female; that occasionally a few or very many of the parthenogenetic eggs develop into males; and that coincident with or, more generally, immediately following the production of male young, many females develop sexual eggs. The sexual egg is visibly and physiologically different from the parthenogenetic egg. When mating occurs, the sexual eggs pass into the ephippium, a specialized, thick-walled covering of the brood chamber, within which they are cast from the mother. These sexual eggs remain dormant for a short or long period, during which the pond may become dried up or solidly frozen. Some of them hatch when favorable water and temperature conditions again arise. They produce female young which, when mature, start the parthenogenetic phase of the cycle again. It has been supposed by many workers—Weismann (1876–79), Woltereck (1911), Papanicolau (1910), and others—that sexual reproduction is entirely or largely due to internal factors, i.e., that there is an innate sexual cycle.

However, in our laboratory cultures of Cladocera, many species may continue by parthenogenetic reproduction for apparently an indefinite period. *Moina macrocopa*, for example, has been reared by exclusively parthenogenetic reproduction for 780 generations and apparently is fully as vigorous as at the beginning of its laboratory

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history. *Daphnia pulex* and other species are also being reared in many different clones. The oldest clones have now been in the laboratory since November, 1911, and have passed through from 666 to 767 parthenogenetic generations without sexual reproduction and without recognized reduction in vigor. These and other breeding data indicate that in at least many Cladocera there are no internal factors which render impossible indefinitely continued parthenogenetic reproduction (Banta, 1914, 1915, 1925). Yet in nature most Cladocera have sexual reproduction, and in laboratory cultures males and sexual eggs occasionally appear. Since male production is apparently not caused by internal factors, it is presumably due to external factors. It was our problem to learn, if possible, what these factors are.

Males were sometimes noted in the laboratory simultaneously in different species and in many different lines having widely different laboratory histories, while at other times for considerable periods no males were encountered in any routine cultures. Likewise in nature males and sexual eggs, when they appear, are usually abundant; and if the pond under observation contains more than a single cladoceran form, all of them ordinarily show sexual reproduction at the same time.

These observations—the lack of apparent need for sexual reproduction; the frequent simultaneous occurrence of males among all cladoceran forms in natural ponds, and in many independent cultures, when males were found in the laboratory—suggested that male (and sexual egg) production were caused by environmental factors. An extensive summary of the occurrence of males in the laboratory stock was made in an attempt to get some information as to the nature of these factors. It was found, by this analysis, that males more frequently occurred in bottles in which three females were cultured than in those containing only one female. Also, we noted that young males commonly appeared in older bottles in which, due to the productivity of the original mother, there were several productive mothers.

This naturally led to the experimental crowding of the mothers with appropriate uncrowded controls. Several species were used in these early experiments (in 1921), namely, *Daphnia pulex*, *D. longi-*

*spina*, *Simocephalus exspinosus*, *S. serrulatus*, *S. vetulus*, *Moina macrocopa*, *M. affinis*, *M. rectirostris*, and *Ceriodaphnia quadrangula*. The results (Banta and Brown, 1923) were substantially the same as those suggested previously for a single species (Grosvenor and Smith, 1913) and showed that the crowded mothers produced a much higher percentage of males than did their uncrowded sisters. The present paper deals with some of the more detailed data for the single species, *M. macrocopa*.

## METHODS AND MATERIAL

Our cultural conditions will be very briefly mentioned. They have been more fully described elsewhere (the early methods in Banta, 1921*b*; and later methods in Banta, 1921*a* and 1925). The culture medium used for a long time was obtained from natural ponds near the laboratory. Since 1916 a manure solution, in which bacteria provide the nutritive element, has been used (Banta, 1921*a*). The latter culture medium is very satisfactory, although it is at times somewhat variable in its physiological effects. Death losses of the animals are infrequent, and reproduction is rapid and prolific. In our stock cultures, newly released young are transferred to wide-mouthed clear-glass bottles of about 200 cc. capacity, each containing approximately 100 cc. of culture water. In each of two bottles is placed a single young female, while three are placed in a third bottle. These bottles are appropriately labeled and examined daily. When young appear, they are at once transferred to a similar series of bottles of the next generation. (For further details of handling, see Banta, 1921*b*). In the sex control experiments, smaller bottles, 150 cc. capacity, of the same type were employed. Approximately 75 cc. of culture medium was used.

*M. macrocopa* was selected for more detailed study, since it is quite amenable to control measures and has a relatively short generation cycle of 2–5 days at temperatures between 20° and 30° C. All of the animals used came from the same clone; and further, all the animals used in a single experiment were sisters of a single brood and were released from their mother's brood chamber within a few minutes.

Ten of these sisters from the same brood were used as a stand-

ard crowded bottle; while one or more sisters, each isolated in separate bottles containing the same amount of culture medium and otherwise receiving the same treatment as the crowded mothers, served as controls. Sexing was done by the microscopic examination of the first antennae of the young animals. In earlier experiments, all the young produced were sexed as soon as feasible after their release. Later, only 100, of the 150 to 250 or more young usually produced in a 10-mother bottle were sexed, inasmuch as tests had shown that a sample of 100 from a crowded bottle gave a very close approximation to the percentage obtained from sexing all the young in the bottle. It was also found that by removing the mothers after the release of their young, sexing could be delayed a day or two, at which time the young animals were older by one instar and could be handled more readily. It was further determined that the first brood produced gave a reliable index of the male production of the mothers. The later broods, produced by the same mother, were subjected to the additional crowding effect of the young previously released. In order to simplify matters, in the later experiments only a single brood, the first brood in the life of the experimental mothers used, was considered.

## EXPERIMENTAL

In all, some 2,900 separate experiments involving crowding of the mothers have been conducted in our attempts to control and to analyze the factors concerned in the control of male production. A few experiments selected to typify the somewhat different results in various experimental series are shown in Table I. These data refer to first broods only. They are typical of those obtained throughout our experiments in which a total of approximately a million young have been sexed.

The 109 uncrowded mothers in the experiments recorded in the table produced 1,954 young, averaging only 0.3 per cent males; 5 males were produced by one mother and 1 by another mother. Even such sporadic male production by uncrowded mothers under normal laboratory conditions is exceptional. Ordinarily, uncrowded mothers produce no males whatever. On the other hand, crowded mothers freely produce males. The 33 moderately crowded bottles (7–14 mothers [usually 10] per bottle), gave an average male

## TABLE I

### Male Production Induced by Crowding the Mothers

| Exp. No. | Date | Control | | | | | Crowded (10± Mothers) | | | | | Heavily Crowded (20± Mothers) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. of Bottles | No. of Mothers per Bottle | No. of Young Sexed | No. of ♂'s | Percentage of ♂'s | No. of Bottles | No. of Mothers per Bottle | No. of Young Sexed | No. of ♂'s | Percentage of ♂'s | No. of Bottles | No. of Mothers per Bottle | No. of Young Sexed | No. of ♂'s | Percentage of ♂'s |
| 1 | 2/22/21 | 2 | 1 | 57 | ..... | 0 | 1 | 7 | 103 | 17 | 17 | 2 | 15 | 214 | 96 | 45 |
| 21 | 4/18/21 | 1 | 2 | 34 | ..... | 0 | 1 | 10 | 106 | 71 | 67 | 1 | 16 | 118 | 64 | 54 |
| 29 | 4/22/21 | 1 | 2 | 50 | ..... | 0 | 1 | 10 | 141 | 81 | 57 | 1 | 20 | 254 | 180 | 72 |
| 30 | 4/22/21 | 1 | 2 | 42 | ..... | 0 | 1 | 10 | 142 | 137 | 97 | 1 | 19 | 205 | 155 | 76 |
| 60 | 8/27/21 | 2 | 1 | 37 | 5 | 13.5 | ..... | ..... | ..... | ..... | ..... | 1 | 19 | 79 | 26 | 33 |
| 69 | 9/ 6/21 | 2 | 1 | 30 | ..... | 0 | ..... | ..... | ..... | ..... | ..... | 1 | 24 | 150 | 106 | 71 |
| 70 | 9/ 6/21 | 2 | 1 | 31 | ..... | 0 | 1 | 14 | 70 | 39 | 56 | ..... | ..... | ..... | ..... | ..... |
| 71 | 9/ 7/21 | 2 | 1 | 35 | ..... | 0 | ..... | ..... | ..... | ..... | ..... | 1 | 19 | 125 | 101 | 81 |
| 74 | 9/ 9/21 | 5 | 1 | 85 | ..... | 0 | ..... | ..... | ..... | ..... | ..... | 1 | 19 | 224 | 31 | 14 |
| 519 | 7/16/23 | 1 | 2 | 39 | ..... | 0 | 1 | 12 | 107 | 43 | 40 | ..... | ..... | ..... | ..... | ..... |
| 520 | 7/16/23 | 1 | 3 | 47 | ..... | 0 | 1 | 14 | 106 | 76 | 72 | ..... | ..... | ..... | ..... | ..... |
| 523 | 7/16/23 | 1 | 2 | 35 | ..... | 0 | 1 | 12 | 102 | 0 | 0 | ..... | ..... | ..... | ..... | ..... |
| 524 | 7/17/23 | 1 | 3 | 41 | ..... | 0 | ..... | ..... | ..... | ..... | ..... | 1 | 15 | 114 | 0 | 0 |
| 525 | 7/17/23 | 1 | 2 | 42 | ..... | 0 | 1 | 13 | 105 | 0 | 0 | ..... | ..... | ..... | ..... | ..... |
| 528 | 7/17/23 | 1 | 2 | 38 | ..... | 0 | ..... | ..... | ..... | ..... | ..... | 1 | 16 | 92 | 8 | 9 |
| 529 | 7/17/23 | 1 | 2 | 38 | ..... | 0 | 1 | 13 | 107 | 7 | 7 | ..... | ..... | ..... | ..... | ..... |
| 535 | 7/18/23 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 1 | 15 | 103 | 103 | 100 |
| 536 | 7/18/23 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 1 | 15 | 105 | 105 | 100 |
| 537 | 7/18/23 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 1 | 16 | 109 | 109 | 100 |
| 538 | 7/18/23 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 1 | 16 | 109 | 109 | 100 |
| 539 | 7/18/23 | ..... | ..... | ..... | ..... | ..... | 1 | 14 | 103 | 93 | 90 | ..... | ..... | ..... | ..... | ..... |
| 540 | 7/18/23 | ..... | ..... | ..... | ..... | ..... | 1 | 13 | 108 | 93 | 86 | ..... | ..... | ..... | ..... | ..... |
| 567 | 7/22/23 | 1 | 1 | 11 | ..... | 0 | 1 | 12 | 104 | 25 | 24 | ..... | ..... | ..... | ..... | ..... |
| 568 | 7/22/23 | 1 | 1 | 12 | ..... | 0 | 1 | 12 | 102 | 10 | 10 | ..... | ..... | ..... | ..... | ..... |
| 591 | 7/25/23 | 1 | 1 | 23 | ..... | 0 | 1 | 13 | 108 | 79 | 73 | ..... | ..... | ..... | ..... | ..... |
| 595 | 7/25/23 | 1 | 1 | 26 | ..... | 0 | 1 | 11 | 115 | 41 | 36 | ..... | ..... | ..... | ..... | ..... |

TABLE I—*Continued*

| Exp. No. | Date | Control | | | | | Crowded (10± Mothers) | | | | | Heavily Crowded (20± Mothers) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. of Bottles | No. of Mothers per Bottle | No. of Young Sexed | No. of ♂'s | Percentage of ♂'s | No. of Bottles | No. of Mothers per Bottle | No. of Young Sexed | No. of ♂'s | Percentage of ♂'s | No. of Bottles | No. of Mothers per Bottle | No. of Young Sexed | No. of ♂'s | Percentage of ♂'s |
| 954 | 6/25/24 | 1 | 2 | 33 | ..... | 0 | 1 | 11 | 119 | 0 | 0 | ..... | ..... | ..... | ..... | ..... |
| 959 | 6/27/24 | 1 | 2 | 35 | ..... | 0 | 1 | 14 | 116 | 89 | 77 | ..... | ..... | ..... | ..... | ..... |
| 960 | 6/27/24 | 1 | 2 | 33 | ..... | 0 | ..... | ..... | ..... | ..... | ..... | 1 | 23 | 129 | 101 | 78 |
| 961 | 6/27/24 | 1 | 2 | 38 | ..... | 0 | ..... | ..... | ..... | ..... | ..... | 1 | 16 | 110 | 89 | 81 |
| 2047 | 11/18/25 | 1 | 2 | 42 | ..... | 0 | 1 | 10 | 103 | 0 | 0 | ..... | ..... | ..... | ..... | ..... |
| 2048 | 11/18/25 | 1 | 2 | 39 | ..... | 0 | 1 | 10 | 103 | 4 | 4 | ..... | ..... | ..... | ..... | ..... |
| 2049 | 11/18/25 | 1 | 2 | 45 | ..... | 0 | 1 | 10 | 124 | 12 | 10 | ..... | ..... | ..... | ..... | ..... |
| 2050 | 11/18/25 | 1 | 2 | 48 | ..... | 0 | 1 | 10 | 116 | 20 | 17 | ..... | ..... | ..... | ..... | ..... |
| 2051 | 11/18/25 | 1 | 2 | 44 | ..... | 0 | 1 | 10 | 106 | 0 | 0 | ..... | ..... | ..... | ..... | ..... |
| 2052 | 11/18/25 | 1 | 2 | 43 | ..... | 0 | 1 | 10 | 122 | 33 | 27 | ..... | ..... | ..... | ..... | ..... |
| 2053 | 11/19/25 | 4 | 2 | 127 | ..... | 0 | 1 | 8 | 116 | 116 | 100 | ..... | ..... | ..... | ..... | ..... |
| 2054 | 11/19/25 | 4 | 2 | 154 | 1 | 0.6 | 1 | 10 | 126 | 101 | 80 | ..... | ..... | ..... | ..... | ..... |
| 2055 | 11/19/25 | 4 | 2 | 119 | ..... | 0 | 1 | 10 | 109 | 92 | 84 | ..... | ..... | ..... | ..... | ..... |
| 2056 | 11/19/25 | 4 | 2 | 124 | ..... | 0 | 1 | 10 | 101 | 70 | 69 | ..... | ..... | ..... | ..... | ..... |
| 2057 | 11/19/25 | 4 | 2 | 108 | ..... | 0 | 1 | 10 | 108 | 108 | 100 | ..... | ..... | ..... | ..... | ..... |
| 2058 | 11/22/25 | 2 | 1 | 45 | ..... | 0 | 1 | 10 | 121 | 9 | 7 | ..... | ..... | ..... | ..... | ..... |
| 2059 | 11/22/25 | 2 | 1 | 35 | ..... | 0 | 1 | 10 | 103 | 30 | 29 | ..... | ..... | ..... | ..... | ..... |
| 2060 | 11/22/25 | 2 | 1 | 46 | ..... | 0 | 1 | 10 | 103 | 17 | 17 | ..... | ..... | ..... | ..... | ..... |
| 2061 | 11/22/25 | 2 | 1 | 43 | ..... | 0 | 1 | 10 | 113 | 0 | 0 | ..... | ..... | ..... | ..... | ..... |
| Totals: | | | | | | | | | | | | | | | | |
| No. of bottles | | 67 | ..... | ..... | ..... | ..... | 33 | ..... | ..... | ..... | ..... | 17 | ..... | ..... | ..... | ..... |
| No. of mothers | | ..... | 109 | ..... | ..... | ..... | ..... | 363 | ..... | ..... | ..... | ..... | 298 | ..... | ..... | ..... |
| No. of young | | ..... | ..... | 1954 | 6 | ..... | ..... | ..... | 3638 | 1513 | ..... | ..... | ..... | 2240 | 1383 | ..... |
| Percentage ♂'s | | ..... | ..... | ..... | ..... | 0.3 | ..... | ..... | ..... | ..... | 41.6 | ..... | ..... | ..... | ..... | 62 |

percentage (3,638 young sexed) of 41.6; only 6 of these bottles failed to produce males; 2, one of which contained only 8 mothers, produced 100 per cent males. The 17 more heavily crowded bottles (15–24 mothers per bottle) averaged (from 2,240 sexed young) 62 per cent males—although 1 bottle failed to produce males; 4 produced 100 per cent males.

These experiments are typical. Male production under normally favorable laboratory conditions is exceptional; while rearing 10 mothers in a bottle results, on the average, in the production of about 42 per cent males; and heavier crowding results in a correspondingly increased male percentage. But while series of heavily crowded bottles frequently average 75–85 per cent males, 100 per cent male production does not often occur in all the heavily crowded bottles of a series.

While crowding the mothers so generally results in male production as to constitute a standard method for securing male production by *Moina macrocopa* and other Cladocera, the results of crowding experiments are by no means uniform. The 45 experiments included in the table were selected not only to illustrate the production of males by crowding but also to show the variable extent to which males are produced by this simple expedient.

For example, Experiments 2053–57 were set up on the same day and in the same culture medium. Numbers 2053 and 2057 produced 100 per cent male young, while the others produced 80, 84, and 69 per cent males, respectively. Experiment Nos. 519, 520, and 523, likewise set up on the same day and from a single jar of culture medium, produced 40, 72, and 0 per cent males. Other series of experiments set up on the same day show similar variation in male production, e.g., Nos. 2047–52 set up November 18, 1925; and Nos. 2058–61 set up November 22, 1925. These are illustrations of the variability in male production within series involving culture medium from a single jar.

Even greater variation in male production is seen when series of experiments set up on consecutive days and from *different* jars of culture medium are compared. While the series set up July 16, 1923 (Nos. 519, 520, and 523), show 40, 72, and 0 per cent males, the series of the following day, July 17 (Nos. 524, 525, 528, and 529),

show 0 per cent males in two crowded bottles and 7 and 9 per cent in the other two crowded bottles. The series started one day later, July 18 (Nos. 535–40), and from a third jar of culture medium, produced 90 and 86 per cent males in 13- and 14-mother bottles, and 100 per cent males in each of two 15- and two 16-mother bottles. Low percentages of males are shown by the experiments set up four days later, July 22; and higher percentages again by those started three days afterward, July 25. Similar differences between series set up on different days and in different culture media are seen in those started November 18, 19, and 22, 1925.

Clearly, differences in culture media are potent factors in influencing the percentage of males produced. These differences are especially marked between experiments given the same treatment but set up on consecutive days and utilizing different jars of culture medium. Even differences arising in the culture medium in individual bottles, set up at the same time, provided with culture medium from a single jar, and handled the same, affect male percentages.

We have no analysis of these culture-medium variations beyond the occasionally different visible appearance or odor of the medium and the differences in percentage of males produced. It is probable that differences in bacterial flora arise and that these (1) through their different excretory products or (2) directly through their different effects as food of the *Moina* or (3) through different effects on the excretory products of the *Moina*, or (4) a combination of these effects, influence the result. While the character of the culture medium is of considerable importance, the amount of available food in a crowded bottle is, at most, a very minor factor in male causation. Among other evidence supporting this latter statement may be cited the fact that in many experiments the mothers that were crowded and produced the usual percentages of males also produced as many young per mother as the uncrowded control mothers which produced only female progeny (Banta, 1925, Table I, p. 54).

The occurrence of variations in male production, under as nearly uniform conditions of crowding as we were able to obtain, makes it clear that crowding per se was not the sole factor contributing to the results. However, in the experiments here considered it was undoubtedly the principal factor, the other factors involved—in addi-

tion to the variations in the composition of our culture medium mentioned above—being temperature and other environmental influences (to be discussed in later papers).

Notwithstanding these variations, the exceeding rarity of male production by uncrowded mothers and the general production of males by crowded mothers clearly indicate that crowding of mothers is in some way causally related to male production. Crowding of mothers is associated with the accumulation of the animals' excretory products. Experiments to be discussed in a later paper indicate that any influence which modifies the excretory products modifies male production. We conclude that the accumulation of excretory products is the potent factor in bringing about male production in crowded bottles.

Two other reports of significant effects of crowding may be referred to. Reinhard (1927) found "that crowding is a very potential, if not the dominant and controlling factor, in stimulating wing development" in the prevailingly wingless cotton aphis. Cobb, Steiner, and Christie (1927) report that with several species of hair-worms, internal parasites of grasshoppers and other insects, the degree of the infestation is directly correlated with the sex of the parasites developing within a given host. Two or three parasites within one host are invariably females; twenty or more are all males; while with an intermediate number there is a sex gradient, the proportion of males increasing with the greater infestation. Excretory products may readily be the determining factor in the former case and are clearly suggested as the factor involved in the latter case.

Several other types of experiments, designed to test out the nature of the effect produced by crowding the mothers, may be mentioned briefly. It was found that 10 mothers, each isolated in a small vial containing 7.5 cc. of culture medium, produced as many males as 10 mothers in 75 cc. of culture medium. This showed that a single mother, as judged by male production, was as much influenced by her own excretory products as by those of other females, provided such products were present in the same concentration.

It was also found that this effect of crowding could be accomplished just as readily by using other genera of Cladocera, *Daphnia* and *Simocephalus*, and in fact with a great variety of aquatic animals

such as *Planaria*, *Asellus*, *Physa*, aquatic insect larvae, small fish, and frog tadpoles. In these experiments, each test mother was confined within a short glass tube, one end of which was placed within the culture bottle containing the other animals. Both ends of the tube were open, but the lower end was covered with tightly drawn silk bolting-cloth. This cloth would, of course, permit of free interchange of solutes between the animals serving as crowders and the female *Moina* confined within the tube. Mothers within similar tubes but without the other animals as crowders served as controls and only rarely produced males. As might be expected from the foregoing experiments, the same result, i.e., male production, was obtained by using either male or female *Moina* as crowders in the culture bottle outside the screened tube. No effect on the sex of the young was found which might be compared to that found in young hermaphroditic *Crepidula* (Gould, 1917) or in *Bonellia* (Baltzer, 1924) unless, indeed, in these latter cases too the amounts of excretory, and not merely female, products are responsible for male development.

## DISCUSSION

Is the production of males by the crowding of the mothers really sex control, or is it, as one geneticist expressed it, merely "the switching off of parthenogenesis and the switching on of sexual reproduction"? Several factors must be borne in mind before we make a definite answer. In the first place, there is no necessary rhythm in the appearance of males and sexual females (Banta, 1914, 1915, 1925; Banta and Brown, 1923), and those writers who refer to environmental agencies as merely hastening or delaying the appearance of sexual forms refer to a condition not found in the majority of Cladocera forms at least. In the second place, the conditions which bring about the production of males and of sexual eggs, while perhaps related in some manner and actually occurring in close sequence in natural ponds, on analysis prove to be different.

Male production occurs in the presence of accumulated excretory products. It occurs in increased percentages with increased crowding, i.e., greater concentration of excretory products. Male production, also, is very slightly, if at all, related to reduced amount of available food, as single mothers in control bottles often produce

no larger broods (all females) than each of the 10 mothers in a crowded bottle (42 per cent males). The rearing of females in dilute food or in clear pond water, even with crowding, usually fails to cause male production or gives a reduced percentage of males.

Sexual egg production, on the other hand, does not occur (except *extremely* rarely) in crowded bottles with the accumulation of excretory products. In fact, we believe accumulated excretory products are not causally related to sexual egg production in *Moina macrocopa*. Sexual eggs are readily produced by female *Moina* reared in clear pond water, and sometimes are produced by individuals reared in very dilute food or very old culture media. Scarcity of food, then, seems the determining factor here, in contradistinction to accumulation of excretory products in the case of male production. The type of treatment (crowding) serves to control the development of the parthenogenetic egg, and may be said to *control sex-determination*.

Cladocera are quite transparent. One may observe the ovarian development of the parthenogenetic eggs of *Moina* for some 40 hours at (20° C.), decide for himself what sex he wishes them to become, and by appropriate treatment cause them to develop into females (control measures for causing female production are 100 per cent effective) or into males (the control with only moderate crowding is about 42 per cent effective on the average). We consider this *control* of sex.

## SUMMARY

1. Having determined that with Cladocera parthenogenetic reproduction may be continued apparently indefinitely; and that when male Cladocera occur, either in natural ponds or in laboratory cultures, different species or stocks having widely different histories frequently produce males simultaneously, we assumed that male production is controlled by external factors.

2. Males occur much more often in our routine cultures in bottles which contain more than a single mother; most often in crowded bottles.

3. Mothers experimentally crowded regularly produce males, while uncrowded controls ordinarily produce only females. On the average about 42 per cent males are produced by 10 mothers reared

in 75 cc. of culture medium. This male percentage can be increased with greater crowding, but does not often reach 100 per cent.

4. A single female, if reared with ample food in a proportionately smaller amount of culture medium, will on the average produce approximately the same percentage of males as 10 mothers reared in 75 cc. of medium.

5. Male production may be equally well controlled by using other species of Cladocera or other aquatic animals as crowders.

6. Male production is apparently very little, if at all, influenced by the *amount* of available food, though it is influenced by the character of the culture medium.

7. Male production in these experiments is associated with the accumulation of excretory products in the culture bottles.

8. This is held to be "control of sex" in a real sense, since conditions for sexual egg production are different from those for male production, and since, further, the experimenter may, by appropriate treatment, determine what will be the sex of the young produced from ovarian eggs already in a late stage of development.

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# CONTROL OF SEX IN CLADOCERA. II. THE UNSTABLE NATURE OF THE EXCRETORY PRODUCTS INVOLVED IN MALE PRODUCTION[1]

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IN THE first paper of this series (Banta and Brown, 1929) it was shown that with the cladoceran, *Moina macrocopa*, isolated parthenogenetic females very rarely produce males among their offspring, but that 10 or more females, when reared in the same amount of culture medium, produce from 40 to 60 per cent males on the average, while with greater crowding of mothers male percentage rises, often to 95–100 per cent. The present paper reports various experiments which were carried out in an attempt to analyze the conditions developing in a bottle crowded with animals, which cause the females to produce male progeny. Disregarding the amount of available food, which was discussed in the paper just referred to, and dismissed as a factor in male production, these experiments naturally fall into three groups: (1) changes in the oxygen and carbon-dioxide tensions of the culture medium, (2) the probably non-volatile nature of the effective excretory products of the animals, and (3) the general transitory nature of the conditions that lead to male production.

## EXPERIMENTAL

### 1. CHANGES IN THE OXYGEN AND CARBON-DIOXIDE CONTENT OF THE CULTURE MEDIUM

One of the first conditions present in a crowded bottle to receive consideration was the probable reduction in the amount of oxygen available to the animals and the concurrent accumulation of carbon dioxide. Normally crowded bottles (10 mothers to 75 cc. of culture

[1] From the Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, New York.

medium), which produce, on the average, 42 per cent males, were then aerated in several ways and were found to produce a greatly reduced percentage of males as compared with their respective crowded control bottles. These experiments involved 118 separate bottles and over 15,500 sexed young. The following types of treatments, which yielded similar results, were employed: (1) exposing the animals in a thin film of water on a slide or in a shallow watch glass several times a day just before egg-laying, (2) the bubbling of crowded bottles with air, (3) the bubbling of crowded bottles with

TABLE I

SUMMARY OF EXPERIMENTS ON AERATION AS INFLUENCING MALE PRODUCTION IN CROWDED BOTTLES*

| Type of Treatment | No. of Experiments | No. of Young Sexed | Control % ♂ | Treated % ♂ | Ratio: % ♂ Treated to ♂ % of Control |
|---|---|---|---|---|---|
| Animals exposed on slide or in watch glass | 16 | 4,828 | 55.9 | 19.5 | 0.35 |
| Bubbled with air | 4 | 993 | 29.2 | 17.2 | 0.58 |
| Bubbled with oxygen and shaken | 9 | 2,126 | 44.7 | 27.7 | 0.62 |
| Bottles shaken | 17 | 5,115 | 37.9 | 24.4 | 0.64 |
| Reared in shallow dishes | 13 | 2,679 | 38.7 | 24.6 | 0.63 |
| Totals and averages | 59 | 15,741 | 43.7 | 23.1 | 0.53 |

* Different types of treatments give the same general result, but apparently the direct aeration of the prospective mothers on a slide or in a watch glass is the most effective method of reducing the percentage of males produced.

oxygen, (4) shaking air into the culture medium every 3 or 4 hours for a time before the eggs are laid, and (5) rearing the animals in the same amount of culture medium but in a shallow dish so that extensive aeration would occur through the large exposed surface of the medium. Table I gives the summaries of these aeration experiments.

A second series of experiments, involving 5,033 sexed young, showed that an increased carbon-dioxide content of the culture medium decreased the male percentage of the crowded and semicrowded (5–7 mothers per 75 cc. of culture medium) bottles very slightly in some series and not at all in other series. These experiments were carried out in two ways. In some of the cases the carbon dioxide was introduced into the test bottles and the bottles tightly corked; in others the gas was slowly bubbled through the culture medium.

When both the carbon dioxide and the oxygen were reduced in amount in the culture medium by bubbling the test bottles with nitrogen, the percentage of males was decreased in crowded bottles and was not increased in semi-crowded bottles. The amount of nitrogen given varied from a lethal amount to that permitting of normal parthenogenetic reproduction.

In some experiments the animals were placed in tightly stoppered bottles with only a small globule of air. In this way the animals would probably reduce the oxygen tension and increase the carbon-dioxide tension of the culture medium. Single mothers were used and placed in about 30 cc. of culture medium. No males were produced.

### 2. THE NON-VOLATILE NATURE OF THE EXCRETORY PRODUCTS OF THE ANIMALS

On the supposition that volatile excretory products might be the effective agent in male production, a series of experiments was planned to attempt to transfer these volatile substances from crowded to uncrowded bottles and thus cause male production in the uncrowded test bottles. The set-up for a single experiment was as follows: A brood of newly released young females was divided among 4–6 bottles (4–7 in each) containing the usual culture water. About one-third of these bottles was set aside to serve as controls; another third was linked in the beginning of a series of 20 or more bottles; and the remaining third of the test bottles was linked at the end of the series. Between the test bottles at the beginning of the series and those at the end of the series were placed 16 or more bottles heavily crowded with *Moina*. The entire series was connected by means of two-holed rubber stoppers and glass and rubber tubing into a closed system. Air was slowly bubbled through the series of bottles from the controls at the beginning of the series to the tests at the end of the series. Obviously the purpose was to transfer any volatile substances from the crowded bottles in the middle of the series to the test bottles in the end of the series. The air was bubbled at various rates, but in most experiments very slowly. The air entering each bottle came in near the bottom in small bubbles through fine capillary tips and passed on from above the surface of the culture medium to the next bottle again through a tube narrowed at its submerged tip. Thus the bottles at the beginning of the series

served as bubbled controls for the test bottles at the end of the series, since they were bubbled to the same extent but with *fresh* air, while the test at the end of the series received only air which had just passed through 16 or more heavily crowded bottles. These experiments showed no greater male production in the bubbled tests than in the bubbled controls. Both tests and controls in the closed series gave fewer males than the other (stoppered) controls not bubbled at all. Obviously the bubbled controls, as anticipated, were somewhat aerated by this treatment and hence produced fewer males than the unbubbled controls; and the bubbled tests were more affected by the consequent aeration than by volatile substances (if any) which they may have received from the heavily crowded bottles.

### 3. TRANSITORY NATURE OF THE CONDITIONS THAT LEAD TO MALE PRODUCTION

Many types of experiments were tried in an attempt to determine the rôle and nature of the excretory products which are influential in causing male production. The first of these involved the introducing of animals, previously uncrowded, into bottles from which large numbers of *Moina* had been removed. Such treatment was effective in causing male production only if the test animals were near the critical period (to be discussed in the next paper of this series) for sex-determination and were introduced soon after the removal of the former occupants of the bottle.

Many slight modifications of the cultural treatment—greater dilution of the food; addition of sodium hydroxide to increase the pH; addition of sulphuric acid to decrease the pH; urea, ammonium hydroxide, and other substances related to excretory products; and several other treatments—almost invariably caused reduction in male percentage in crowded or semicrowded bottles as compared with their respective controls. Various combinations of the foregoing treatments also produced the same results. These treatments were made very exhaustively, the amount of treatment ranging from lethal dosages to those having a just perceptible effect. The male percentage of the treated bottles was, on the average, 10–90 per cent of that shown by the untreated controls (Table II). In all, about 225 experiments, involving the sexing of over 44,000 young, were carried out; but only those experiments having some males in either control

or test are included in this table. One treatment, that with uric acid, failed to show any appreciable effect in decreasing male production, and in several experiments actually seemed to increase slightly the number of males produced by the test mothers.

## DISCUSSION

Grosvenor and Smith (1913), working with *Moina*, concluded that the effective agent in causing the production of males in crowd-

TABLE II

SUMMARIES OF SOME EXPERIMENTS ON THE CONTROL OF SEX IN *Moina macrocopa* WITH SUBSTANCES RELATED TO EXCRETORY PRODUCTS AND OF EXPERIMENTS ATTEMPTING TO MODIFY THE CONCENTRATION OF HYDROGEN IONS*

| Type of Treatment | No. of Experiments | No. of Young Sexed | Control % ♂ | Treated % ♂ | Ratio: % ♂ Treated to ♂ % of Control |
|---|---|---|---|---|---|
| $CO_2$ | 26 | 5,033 | 41.7 | 37.3 | 0.89 |
| Uric acid | 11 | 2,069 | 10.6 | 11.4 | 1.07 |
| $CO_2$ and uric acid | 28 | 4,964 | 23.3 | 7.7 | 0.33 |
| Urea | 16 | 2,743 | 14.3 | 9.5 | 0.66 |
| $NH_4OH$ | 17 | 3,655 | 40.1 | 20.2 | 0.50 |
| Urine | 25 | 5,739 | 27.9 | 11.1 | 0.39 |
| Chicken manure | 6 | 1,239 | 15.5 | 1.3 | 0.08 |
| Reared under increased air pressure | 23 | 6,194 | 27.6 | 24.8 | 0.90 |
| $CO_2$ under pressure | 16 | 2,464 | 24.5 | 22.4 | 0.91 |
| NaOH | 6 | 1,465 | 17.4 | 15.7 | 0.90 |
| $H_2SO_4$ | 10 | 1,858 | 21.7 | 13.7 | 0.63 |

* The percentages of males produced by the controls is lower than the average for crowded bottles. This is due chiefly to the inclusion of many experiments utilizing semicrowded bottles.

ed bottles was the accumulation of the excretory products of the animals. They also tried the evaporation of the culture water in which many *Moina* had been kept for a time and the introduction of the dry residue into other culture bottles. This expedient failed to stimulate male production. They concluded that the excretory products responsible were of an unstable nature.

The various experiments reported in the present paper and in the earlier paper of this series (Banta and Brown, 1929) support the conclusion that excretory products are causative factors in male production. The effective elements of these excretory products apparently have an unstable nature and are very transitory in effect, as is shown by the following observations: (1) bottles of culture media

which have had large populations of *Moina* retain for a very limited period their ability to cause male production by other females, (2) the number of treatments which cause a breaking-down or disruption of the balance of excretory products responsible for male production is large and quite diverse. Only one treatment used, uric acid, failed to have an appreciable effect in destroying or neutralizing these effective products. The diversity of treatments which caused decreased male production in crowded bottles perhaps points to the possibility that any treatment which disturbs the balance of accumulated excretory products reduces male production.

The series of experiments designed to test the possible volatile nature of the excretory products gave negative results. Whether the tests, bubbled gently with air previously bubbled gently through several crowded bottles, were likewise too much aerated; or whether the substances involved in male production in crowded bottles are not readily volatile and hence are not transferred in this manner was not determined. The amount of bubbling was very slight in several of these experiments, and it is strongly suspected that the excretory substances involved are not readily volatile but are (in part, at least) unstable and are broken down by even limited aeration. At any rate, we were unable to transfer the potent excretory substances from one bottle to another and thus demonstrate their volatility.

## SUMMARY

1. The accumulation of excretory products is favored as the principal factor in stimulating male production in the experiments discussed. This statement is consistent with all the experimental evidence.

2. The excretory products concerned in male production, while apparently non-volatile, are presumably quite unstable and easily broken down or neutralized by a diversity of secondary conditions, and thus easily lose their potency in controlling sex-determination in Cladocera.

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# EXPERIMENTAL MODIFICATION OF NERVE DEVELOPMENT IN AMBLYSTOMA

(Seventeen figures)

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AN EVER increasing body of evidence supports the view that the form, size, and distribution of nerves can be modified by changing conditions in the tissue environment, outside of the central nervous system, in which the nerve develops. The axone is an outgrowth of the neuroblast, but the path it takes through tissues seems to be largely a product of its reactions to its environment at each stage of its progress. The analysis of these environmental factors in the development of a nerve has not proceeded much beyond indicating the general truth of the proposition, which is illustrated, for example, by Detwiler's (1920, 1922, 1925) demonstration of a certain specificity in the "attraction" exerted by the anterior limb bud of *Amblystoma* on the nerves of the brachial plexus. With certain limitations the third, fourth, and fifth spinal nerves furnish the major part of the innervation of the fore limb, even though the limb be located a considerable distance posterior to its normal site, the nerves under these conditions deviating from their normal course in order to effect innervation. There are limits, however, to this specificity. Thus, when a differentiating limb bud is implanted at the level of the seventh, eighth, and ninth segments, it may receive the fifth, sixth, and seventh nerves instead of the normal third, fourth, and fifth. Under these conditions nerves not normally involved in the brachial plexus are "attracted" to or by the limb bud. As Detwiler (1926) has suggested, the apparent reason why the third and fourth nerves fail to make connections with the limb under these conditions is that any stimulus from the limb bud is too far removed posteriorly to be effective on nerves growing out from the higher levels.

In the experiments herein reported, which were performed in the spring of 1926, the publication of which has been delayed through pressure of other work, the right limb bud of *Amblystoma punctatum*,

at the tail-bud stage, was excised and reimplanted at varying distances caudad to its normal position, and the right eye and part of the nasal placode of another embryo of the same age were grafted in the limb site. The purpose of the experiment was twofold: (1) to note the effect on the normal limb nerves produced by strange cells, actively growing and differentiating in the normal limb site, and (2) to study the development of the optic nerve under these conditions. The transplanted limb in some cases was so far removed from the sphere of action of the normal brachial nerves as to be practically absent so far as these nerves were concerned; in other cases it was close enough to the normal limb site to receive innervation from the normal limb nerves as well as from more posterior ones.

The operation was easily carried out and some forty successful transplants were readily obtained. Most of these were allowed to develop until rather late larval stages. Five specimens of different ages and representative of as many conditions were selected for study and sectioned.

The larvae were killed and fixed according to the method of vom Rath and imbedded in rubberized paraffin. They were cut in nearly every case as far back as the transplanted limb in sections 10 micra thick.

Graphical reconstructions were made of the portions of the larvae concerned in the experiment. In some cases reconstructions were made of the normal side as well as the transplant side for the sake of comparison. Right and left sides are in reverse positions in the reconstructions. Cell counts were made both of normal ganglia and of ganglia on the transplant side, and compared. The animals were sketched before sectioning, to show the general topography.

## RESULTS

In all cases the transplanted eye developed just opposite the left limb in the right limb site. In all cases a portion or all of the nasal placode was transplanted with the eye, and developed more or less fully. In all cases but one, a portion of the forebrain was transplanted with the eye and developed as one or more nerve masses or knots near the eye.

Table I shows comparative spinal ganglion cell counts for the

normal and transplant sides. The left side in all cases is the normal. The vertical dotted lines connect the arrows which represent nerves from the corresponding ganglia that take part in the formation of a brachial plexus.

TABLE I

COMPARISON CHART OF GANGLIA COUNTS*

| GANG. NO. | CASE NO. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LE 1 | | LE 44 | | LE 27 | | LE 21 | | LE 24 | |
| | Right | Left | Right | Left | Right | Left | Right | Left | Right | Left |
| II | 905 | 943 | ..... | ..... | 372 | 605 | 746 | 688 | 357 | 627 |
| III | 681 | 1,992 | → 1,202 | 936 | 1,169 | 1,420 | 840 | 1,445 | 649 | 1,441 |
| IV | 1,011 | 1,954 | → 1,611 | 1,960 | → 1,513 | 1,397 | → 1,235 | 1,210 | 961 | 1,461 |
| V | 1,022 | 1,225 | → 1,813 | 2,140 | → 1,193 | 965 | → 1,155 | 1,055 | 874 | 1,033 |
| VI | ..... | ..... | 824 | 1,092 | → 988 | 659 | → 808 | 591 | → 1,123 | 724 |
| VII | ..... | ..... | → 1,736 | 570 | ..... | ..... | → 891 | 663 | ..... | ..... |
| VIII | ..... | ..... | → 63 | 959 | ..... | ..... | → 786 | 380 | ..... | ..... |
| IX | ..... | ..... | ..... | ..... | ..... | ..... | → 620 | 493 | ..... | ..... |

* Nerves VIII and IX in LE 21 innervate the limb independently and do not join the plexus. Limbs of LE 1, LE 24, and LE 21 (duplicated hand), non-functional. LE 27, duplicated limb, both components functional. LE 44, normal and functional. Dotted lines and arrows indicate the brachial plexuses.

TABLE II

COMPARISON CHART OF GANGLIA COUNTS ON THE PERCENTAGE BASIS*

| GANG. NO. | CASE NO. | | | | |
|---|---|---|---|---|---|
| | LE 1 | LE 44 | LE 27 | LE 21 | LE 24 |
| II | 0.958 | ..... | 0.614 | 1.084 | 0.561 |
| III | 0.341 | → 1.284 | 0.823 | 0.581 | 0.450 |
| IV | 0.517 | → 0.821 | → 1.082 | → 1.020 | 0.657 |
| V | 0.834 | → 0.847 | → 1.236 | → 1.094 | 0.846 |
| VI | ..... | 0.754 | → 1.484 | → 1.365 | → 1.551 |
| VII | ..... | → 3.045 | ..... | → 1.343 | ..... |
| VIII | ..... | → 0.065 | ..... | → 2.068 | ..... |
| IX | ..... | ..... | ..... | → 1.257 | ..... |

* As in table I, the ganglia supplying the brachial plexus are indicated with arrows joined by dotted lines.

Table II is another comparison chart of ganglia counts, representing the quantitative facts of the chart in Table I on a percentage basis. The left or normal ganglion is in each case considered 1.0, and the relative size of the right ganglion is determined in decimal fractions. Only the values for the ganglia on the transplant side are

given in the table. It must be borne in mind, however, that such a chart cannot give more than approximate information, since the left ganglion, which is, of course, subject to the influences which cause variations in all normal tissues, is taken as a standard.

## DESCRIPTION OF CASES

CASE LE 1.—The transplanted eye developed in the limb site (Fig. 1). All parts of the eye are present, and the transplanted eye

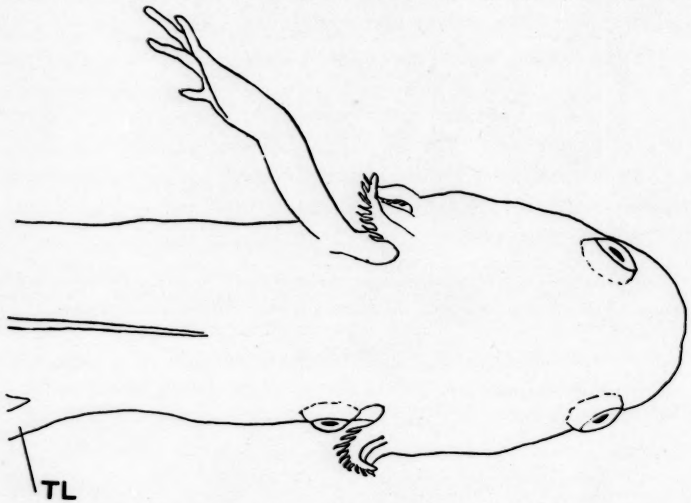


FIG. 1.—LE 1. Operated April 15, killed July 20, 1926. Transplanted limb normal in structure but functionless. ×6. *T.L.*, transplanted limb.

is of approximately the same size as the normal eyes. Where the optic nerve grows out, there is a fingerlike evagination of the retina into the tissue of the host, shown in Figure 2 and at *A* in Figure 3. The extent of the evaginated portion is approximately two-thirds of the distance across the eye. The pigmented layer of the retina has not remained compact, but portions of its peripheral surface seem to have sloughed off and become scattered through the surrounding tissue. This is especially true near the most medial region of the evaginated retina. There is no connection apparent between the evaginated retina and any of the spinal nerves. These facts are clearly shown in the photograph, Figure 2.

Evidently a portion of the forebrain was transplanted with the eye, since a clump of nerve tissue has developed which begins near the outgrowth of the optic nerve and extends posteriorly a distance of 1.42 mm. The eye itself measures 1.5 mm. from anterior to posterior extremities. The optic nerve, after leaving the evaginated portion of the retina, joins the clump or knot of nerve tissue which lies adjacent to the eye at this point, and becomes inextricably interwoven with the cells and fibers of this knot. In Figures 2 and 3, *B* marks the approximate beginning of the knot.

FIG. 2.—Photograph of a section of eye of LE 1, showing evagination of retina, and optic nerve at *A* in the evagination. *B* marks the beginning of the nerve mass into which the optic nerve enters later. The photograph shows how the nerve mass and some mesenchyme tissues are surrounded by pigmented material.

May and Detwiler (1925) described a case (EE 10) in which a similar distortion of the eye has occurred where the optic nerve grows out. In that case, the optic nerve was seen to make a connection with the IX–X ganglionic complex. This phenomenon is attributed to an attracting force proceeding from the nerve center, "in this case the IX–X ganglionic complex, which brings about the local deformation of the eye where it joins with it" (p. 92).

As stated above, there is no indication in case LE 1 of any connection between the optic nerve and the central nervous system. It becomes apparent then that a protuberance as described by May and Detwiler and as exemplified in case LE 1 must be the result of some other cause than the attractive influence of the nerve center.

The optic nerve joins the large nerve knot described. In further descriptions of other embryos, it will be noted that the optic nerves join such nerve knots, but in no other case was such distortion noted. It can be concluded that the nerve knot is not reponsible for the described evagination.

Midway between the evaginated portion of the eye and its posterior margin, the cartilage around the eye is perforated. The perforation is seen to be complete in two consecutive sections only, while

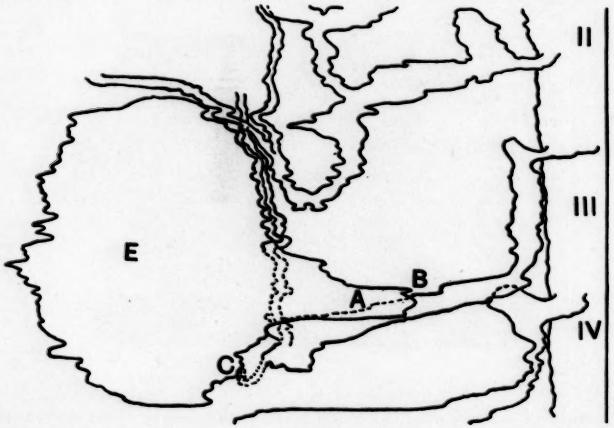


FIG. 3.—Reconstruction of LE 1, showing paths of nerves in relation to the eye. *A* marks the evaginated portion of the retina, and *B* the approximate beginning of the nerve knot into which the optic nerve enters. At *C* a branch of the third spinal nerve perforates the cartilage around the eye, but an intimate connection with the eye is not in evidence. *E*, eye.

those immediately following show incomplete perforation. A bit of tissue, which it is difficult to identify as nerve tissue, extends through this perforation but not beyond the cartilage in the direction of the retina. Fibers of this tissue are not seen to penetrate the retina of the eye but can possibly be demonstrated as being a portion of the third spinal nerve (Fig. 3 *C*).

A small portion of the nasal placode was transplanted with the eye and has developed in a limited measure just ventral to the transplanted eye. There is no olfactory nerve, nor is there a connection between spinal nerves and the nasal sac.

An examination of the retina shows that all the layers are present. The outer layers are somewhat separated on account of shrinkage. Laurens and Williams (1917) found that the retina of transplanted eyes contains fewer ganglion cells than the normal. Though no cell count was made in these cases, it is easy to see that the ganglion layer of the retina is deficient. In the transplanted eye of LE 1 there is generally but one layer of ganglion cells, whereas the normal contains two layers. The result of fewer ganglion cells is that the optic nerve, whose fibers arise from them, is smaller than the normal.

The second spinal nerve divides not far from the spinal ganglion. The anterior branch unites with the first spinal nerve. The other branch descends and travels caudad toward the eye, whence it is deflected cephalad where it divides again, one part to join a more peripheral portion of the first spinal nerve, the other to supply surrounding muscular structures.

The third spinal nerve here forms no part in the plexus supplying the limb. The nerve approaches the eye and divides there, one branch, like the branch of the second, being deflected cephalad, and dividing presumably to supply the periphery and the muscular structure in the region anterior to the transplant (Fig. 3).

Unfortunately, the sections in this case extended no farther caudad than just posterior to the fifth spinal ganglion, and some of these were torn. Thus it was not possible to ascertain whether or not the fourth and fifth nerves join in a plexus or whether they innervate the limb.

A comparison of nerve-cell counts in the ganglia of the normal side and the side containing the transplant shows that the second spinal ganglion has not been noticeably affected by the transplant (see Table I). There is, however, a marked sensory hypoplasia in the third spinal ganglion on the transplant side. This is in keeping with the findings of Detwiler (1923), who showed that removal of the limb rudiment causes a hypoplastic development in the ganglia which normally supply the limb with sensory neurones if the rudiment is removed prior to the outgrowth of these neurones. Although in this case, the limb has not been entirely removed but merely placed caudad to its normal position, the third spinal nerve does not

contribute toward its innervation. Since it innervates neither the limb nor the eye, its normal sensory field is reduced and hypoplastic development, as it does occur, would be expected.

The third spinal ganglion is 0.341 of the normal (see Table II). The fourth and fifth ganglia are not as deficient, but nevertheless

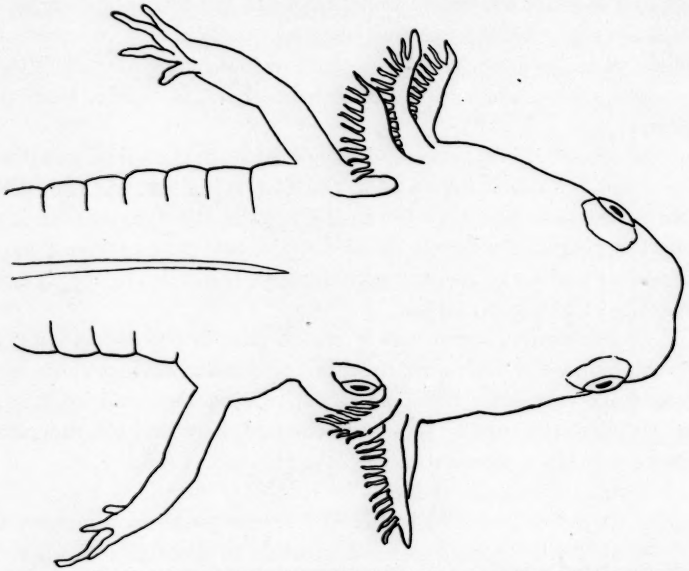

FIG. 4.—LE 44. Operated April 29, killed July 21, 1926. Normal movements in transplanted limb. ×6.

they show marked hypoplasia when compared to the normal, the fourth being 0.517 and the fifth being 0.834 of the normal.

*Summary.*—The transplanted eye, developed in the limb site, shows a distortion where the optic nerve grows out to join an adjacent nerve knot. There is no demonstrable connection between spinal nerves and the optic nerve of the transplanted eye. The third, fourth, and fifth spinal nerves show hypoplasia, evidently due to the removal of the normal sensory stimulus.

CASE LE 44.—The transplanted eye developed opposite the third, fourth, and fifth spinal segments (Fig. 4). The general appearance of the transplanted and normal eyes is approximately the same.

The pigment layer is separated from the nervous layer in both, probably on account of shrinkage. The ganglion cells of the retina in the normal eye are found along the inner margin of the reticular layer, whereas the ganglion cells of the transplanted eye are found to lie within the reticular layer itself. This layer obscures the cells so that it is difficult to ascertain whether or not there are fewer cells in the ganglion layer of the transplant (Fig. 5).

The optic nerve of the transplanted eye is much smaller than the normal. It penetrates the bulb of the retina and, as it emerges, becomes lost in a nerve knot which has formed between the nasal placode and the eye (Fig. 5, Fig. 6 *O*).

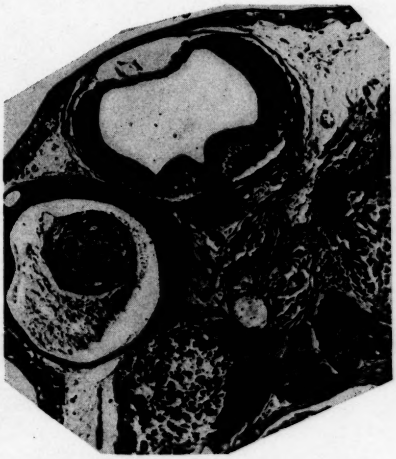

FIG. 5.—Photograph of section of LE 44, showing the transplanted eye and the adjacent nasal sac. The optic nerve is seen entering the nerve knot adjacent to the eye. The three lobes of the dorsal pancreas are seen opposite the eye and nasal sac.

The nasal sac, which has developed more fully in this case than in the others examined, lies ventral to the eye opposite its caudal half (see reconstruction, Fig. 6). Along the interior margins of the nasal sac and the eye and in the angle between them is the nerve knot into which the optic nerve enters. Its size is about one-fourth the nasal sac. This knot is innervated by a branch of the third spinal nerve, which through the knot penetrates the nasal sac (*A* in Fig. 6), and by a branch of the vagus which penetrates it midway between its anterior and posterior limits (*V* in Fig. 6). The branch of the vagus which thus penetrates the knot leaves the vagus nerve at a point a little anterior to the third spinal segment. It passes through the knot and emerges on the opposite side, ramifying into peripheral tissue.

Thus it is seen that three different nerves enter the knot between

the nasal sac and the eye, namely, the third spinal nerve, the branch of the vagus, and the optic nerve. However, they enter it at different levels, so that they do not seem to interfere with each other at any time. The third spinal nerve enters most anteriorly. The optic nerve enters between the anterior margin of the knot and the middle, while the branch of the vagus enters near the middle.

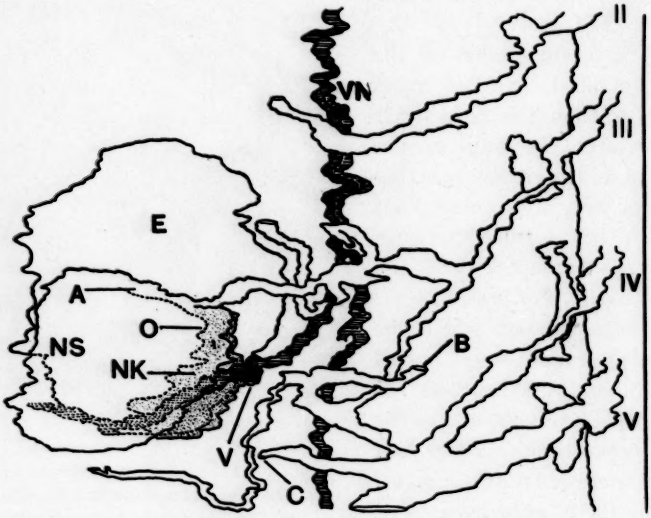


FIG. 6.—Reconstruction of LE 44. *A*, entrance of branch of third spinal nerve into nasal placode. *B*, branch of nerve entering dorsal pancreas. *C*, branch of nerve entering a nerve knot. *E*, eye. *N.K.*, nerve knot. *N.S.*, nasal sac. *O*, point of entrance of optic nerve into nerve mass. *V*, branch of vagus nerve passing through nerve mass. *V.N.*, vagus nerve.

Near the posterior portion of the eye, the cartilage is perforated, and fibers from the knot penetrate the cartilage for several consecutive sections. But none of these fibers is seen to penetrate the retina.

Another large nerve knot is formed just caudad to the region of the transplant and receives fibers from a branch of the fifth spinal nerve; or perhaps fibers from the third, fourth, and fifth spinal nerves combined, since they form a plexus before this branch is emitted (*C* in Fig. 6).

A branch of the third spinal nerve enters a large glandular structure found in the muscular tissue near the eye. Three areas of this glandular tissue are seen for many consecutive sections, whereupon they fuse and gradually slip into the coelom where the gland lies dorsal to the intestine. The gland empties into the intestine by two distinct ducts. These ducts and the general appearance of the gland are responsible for the inference that it is the dorsal pancreas. The reason for its anterior growth into mesenchyme tissue and its innervation by a spinal nerve, however, is not apparent. The point of entrance of the spinal nerve into the gland is shown by *B* in Figure 6.

The second spinal nerve is not affected by the transplant. On the unoperated side, the second ganglion is diffuse and pushed out of its normal position. The nerve cells are scattered and lie among nerve fibers so that they could not be counted with accuracy. Therefore no comparison of the ganglia counts is given for the second spinal ganglia in this case.

Besides innervating the above mentioned structures, the third spinal nerve joins the fourth and fifth to form a plexus. The plexus is increased in size by the fact that the fifth spinal nerve joins with the seventh and the eighth. Thus the limb is innervated by a plexus composed of the third, fourth, fifth, seventh, and eighth nerves. The limb itself is opposite segments VIII, IX, and X. One branch of the fifth nerve enters a large nerve knot caudal and medial to the eye.

The third spinal nerve on the transplant side shows marked sensory hyperplasia. On the ground that increased sensory stimulus at the periphery causes such hyperplasia (Detwiler, 1923), this phenomenon could be said to be the result of the fact that one branch innervates the nasal sac and another the anteriorly grown dorsal pancreas. Both these areas afford a stimulus foreign to and possibly greater than the normal. Detwiler (1928) notes the penetration of the fifth spinal nerve into the nasal sac, in embryos in which the limb bud had been removed and the eye and nasal placode grafted three or four segments caudad to the normal limb site, but gives no data on the effect on spinal ganglia.

The fourth and fifth spinal nerves on the transplant side both show hypoplasia. They evidently do not have the normal amount of

sensory stimulus from the periphery. The amount of hypoplasia in the fifth nerve, which enters the nerve knot, is not quite as great as that in the fourth (see Tables I and II). The sixth and eighth ganglia both show hypoplasia. The sixth does not join the plexus to the limb, but the seventh and eighth do. The reason why the

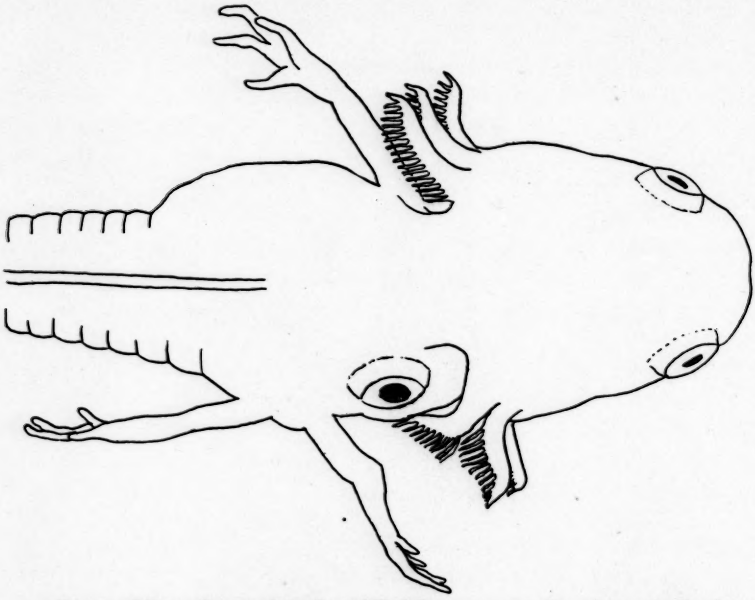

FIG. 7.—LE 27. Operated April 23, killed June 23, 1926. Complete reduplication in transplanted limb; both parts functional. ×10.5.

seventh should show hyperplasia and the eighth such unusual hypoplasia is not apparent.

*Summary.*—The transplanted eye is nearly normal in appearance. The optic nerve enters a nerve knot between the eye and nasal sac. There is no evidence of a connection between spinal nerves and the transplanted eye. One branch of the third spinal nerve enters the nasal sac and another branch the anteriorly grown dorsal pancreas. A branch of the fifth enters a large nerve knot near the eye. The third, fourth, fifth, seventh, and eighth nerves form the brachial plexus. Of the brachial plexus the third ganglion shows hyperplasia;

and the fourth and fifth, hypoplasia. A branch of the vagus nerve passes through the nerve knot between the nasal sac and the eye.

CASE LE 27.—The transplanted eye developed opposite the third, fourth, and fifth spinal segments (Fig. 7). All the parts of the normal eye are present. Anterior to the eye a small nasal sac has developed, on the medial margin of which a small nerve knot is attached. Between the eye and the central axis of the larva, a very large knot extends through the region opposite the posterior part

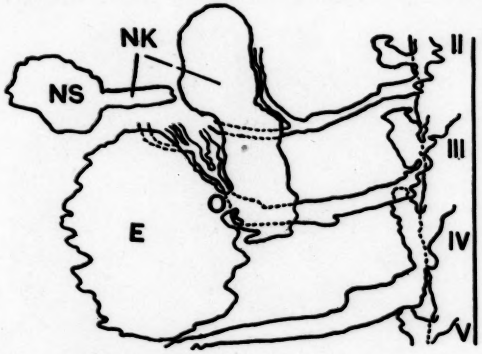


FIG. 8.—Reconstruction of LE 27. *E*, eye. *N.K.*, nerve knot. *N.S.*, nasal sac. *O*, point of entrance of optic nerve into nerve knot.

of the first, the second, third, and anterior part of the fourth spinal segments (Fig. 8).

The transplanted eye shows a deficiency in the number of ganglion cells. The optic nerve is smaller in the transplant than in the normal eyes. After penetrating the retina, the optic nerve enters the large nerve knot described in the paragraph above. There are no connections between the nerve knot and the spinal nerves (Fig. 8).

The second spinal nerve sends a small branch to join the first and also one to join the third nerve. A ganglion count for this nerve shows its size to be little more than half of the normal; the reason for this is unknown. The third spinal nerve grows laterally until it encounters the eye (Fig. 9), whence it is deflected cephalad along the eye and then toward the periphery, where it branches. The third spinal ganglion shows hypoplasia, having a cell count of 1,169 against

the normal 1,420. This is to be expected since the sensory stimulus at the periphery has been reduced. The fourth, fifth, and sixth nerves innervate the limb which is opposite the sixth, seventh, and eighth segments. The fourth, fifth, and sixth ganglia show hyperplasia (see tables)—the fourth and fifth probably from an added burden caused by the absence of the third nerve from the plexus; and the sixth, because of the additional foreign sensory stimulus.

*Summary.*—The optic cup developed in the limb site, and the optic nerve enters a large nerve knot adjacent to the eye. The third

FIG. 9.—Photograph of section of LE 27, showing how closely the third spinal nerve approaches the eye before being deflected. The nerve is the black diagonal line almost touching the eye. There is no connection between the nerve and the eye.

spinal nerve approaches the eye and is deflected cephalad. The third spinal ganglion shows hypoplasia. The fourth, fifth, and sixth spinal nerves innervate the limb and the corresponding ganglia show hyperplasia. There is no connection between spinal nerves and the transplant.

CASE LE 21.—The transplanted eye developed in a position opposite the third and fourth spinal segments (Fig. 10); the normal constituents of the eye are present. The eye is covered with a layer of cartilage, which is not of uniform thickness and which is absent in some places. Though spinal nerve fibers approach these regions more or less unprotected by cartilage, none of them penetrates the retina. In the caudo-ventral three-fifths of the eye, a nerve knot has

formed between the nervous and pigment layers of the retina. As a result, the nervous layer of the retina has been forced inward, into the cavity of the eye, to provide room for the knot. Nor has the pigment layer of the retina remained in place. The amount of outward bulge caused by the knot is an approximate counterpart of the inward bulge of the nervous layer (Fig. 11). There are no connections between the central nervous system and the knot. Caudad the knot protrudes from the eye and is covered with a layer of cartilage. A small optic nerve enters the nerve knot and becomes lost in it.

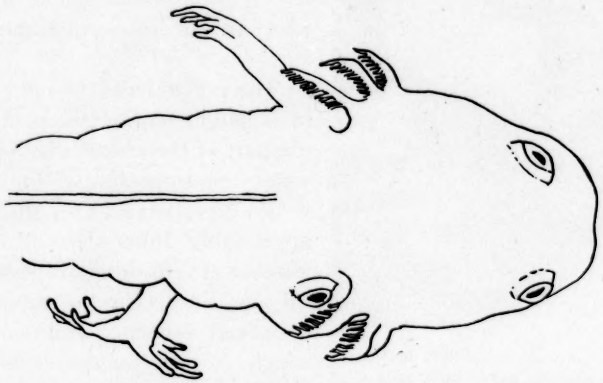

FIG. 10.—LE 21. Operated April 22, killed June 29, 1926. Partial reduplication in transplanted limb; non-functional. ×6.

A small and very incomplete nasal sac developed ventrally and caudad to the transplanted eye. This is no doubt due to the fact that only a portion of its rudiment was transplanted. There seems to be no supply of nerves to the nasal sac.

The transplanted limb developed opposite spinal segments VIII, IX, and X. It is innervated by a plexus formed by the fourth, fifth, sixth, and seventh spinal nerves, and by the eighth nerve independently (Fig. 12). Whether or not the ninth nerve contributes toward the innervation could not be definitely ascertained, since the animal was not sectioned that far; but it is probable because the corresponding ganglion shows hyperplasia (Table I).

The first and second spinal nerves are normal in position, and a ganglion cell count for the second spinal ganglion shows approxi-

mately as many cells on the transplant side as on the normal—746 transplant, 688 normal, a difference of only 58 cells, which may be considered of no special significance. The third spinal ganglion on the transplant side contains only 840 cells, while that on the normal contains 1,445—a difference of 605 cells, more than 50 per cent of the ganglion cells on the transplant side. The nerve from this hypoplastic ganglion is small and, unlike the normal, takes no part in the formation of the brachial plexus. It approaches the eye, passes beside it for a distance, and then its fibers spread into surrounding tissue.

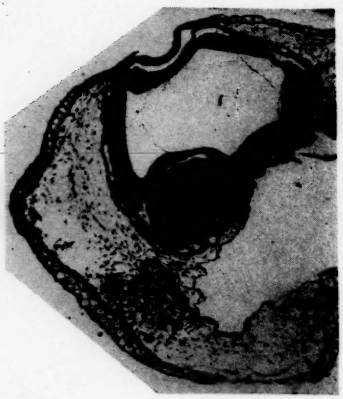

FIG. 11.—Photograph of section of LE 21, showing nerve knot as a solid black mass within the retinal layers of the eye. The entrance of the optic nerve into the knot is not shown.

The nerves from the fourth and fifth ganglia on the transplant side are part of the plexus which innervates the transplanted limb. As would be expected, they differ inappreciably from the normal in number of cells and in topographical size, since they are subject to a normal influence from a limb which is nevertheless remote in position.

Furthermore, the sixth, seventh, and eighth ganglia show marked hyperplasia, confirming the view that increased functional demands at the periphery result in increased development of the sensory nerve centers. The eighth ganglion furnishes a nerve supply to the limb but does not join the plexus. The hyperplasia in this ganglion is much more marked than in the sixth and seventh. The ninth ganglion also shows hyperplasia; and although its peripheral fibers could not be traced, one could assume that it assisted in the innervation of the limb because such increased sensory demands cause hyperplasia in other cases.

*Summary.*—A nerve knot has developed within the eye itself, between the pigment and nervous layers of the retina. There are no connections between spinal nerves and the knot or the eye. The

optic nerve enters the knot. The fourth and fifth spinal nerves join a plexus to innervate the limb, and their ganglia are practically normal. The third spinal nerve approaches the eye and is deflected for-

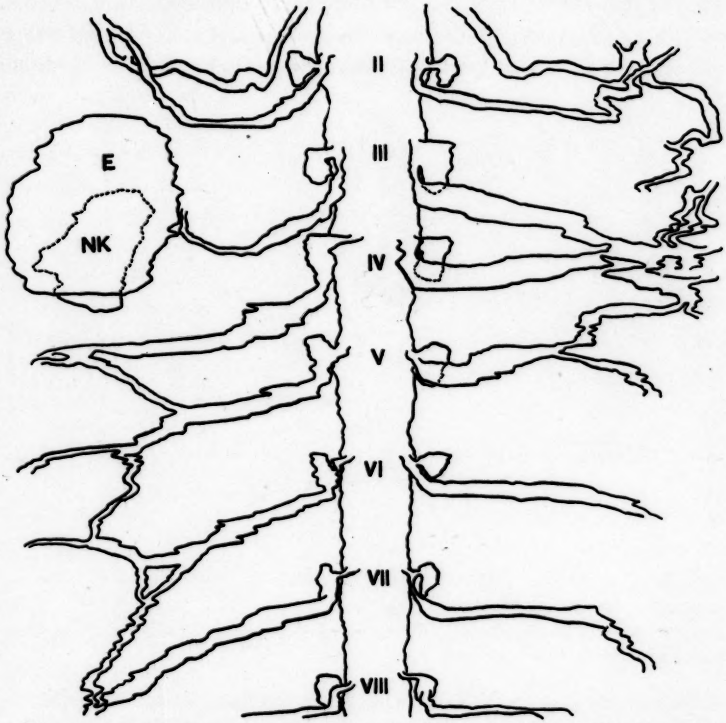


FIG. 12.—Reconstruction of LE 21. *E*, eye. *N.K.*, nerve knot. This figure illustrates the preference of the nerves at the limb-level for the remotely situated limb and also the innervation of the limb by nerves from segments opposite its new position. However, the limb is not functional.

ward, and its ganglion shows hypoplasia. The sixth, seventh, eighth, and ninth ganglia show hyperplasia.

CASE LE 24.—The transplanted eye occupies a position approximately opposite the third and fourth spinal segments (Fig. 13). The optic nerve does not penetrate the retina at any point in the eye. The optic nerve is, however, present and seemingly divided into a

number of smaller nerves, which do not seem to have a common origin. These portions of optic nerve are seen along the inner margin of the ganglion cell layer of the retina in almost any part of the eye (Fig. 14).

Laurens and Williams (1917), in their experiments on eye transplantation, state that the "optic nerve has also never been observed

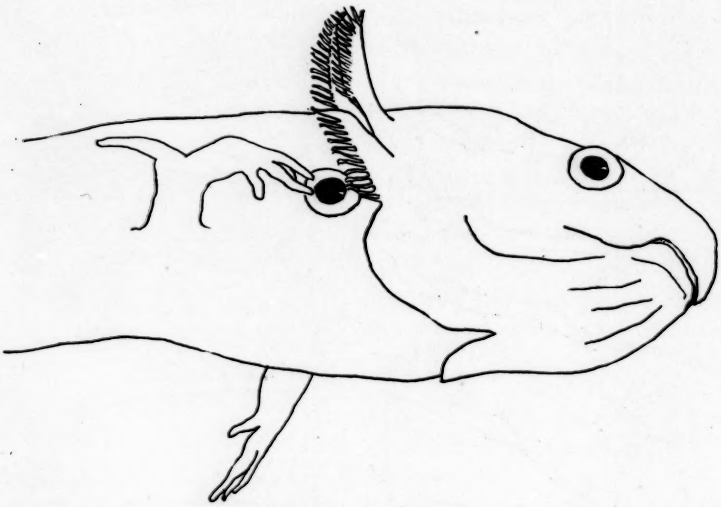

FIG. 13.—LE 24. Operated April 22, killed June 23, 1926. Partial reduplication in transplanted limb; non-functional. ×6.

to be present" (p. 72). From their description it is not possible to ascertain whether or not this is an analogous case; for though the optic nerve in its normal position and contour is not present in LE 24, its fibers are present nevertheless. Lewis (1907) describes another anomaly in the outgrowth of the optic nerve. "In a few instances the nerve instead of passing through the retina extends directly from the ganglionic layer across the cavity of the eye and through the pupil into the mesenchyme" (p. 265). In neither of the instances cited are the attendant circumstances which accompany the abnormal growth or absence of the optic nerve described.

The retina of LE 24 shows, like those of the other larvae de-

scribed, a deficiency in the number of ganglion cells; and the other cell layers are much less neatly arranged than in the normal (Figs. 14, 15).

A number of spinal nerves approach the eye, but none is seen to penetrate it (Figs. 16 and 17). In a number of places the cartilage surrounding the eye is discontinued and the tissue of the eye is exposed to the tissue of the host without showing any signs of intermingling. However, at one point, the muscle tissue, presumably of the host, and the cartilage of the transplant have united (*A* in Fig. 16).

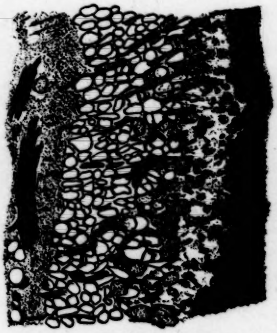

FIG. 14.—A section through the retina of the transplanted eye of LE 24. The layers are less regularly arranged than in the normal. The portions of the optic nerve remain scattered along the inner margin of the retina, not growing through the bulb at any point.

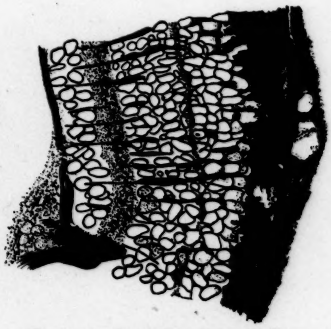

FIG. 15.—A section through the normal retina of LE 24 at the point of formation of the optic nerve.

Opposite the more anterior portion of the eye and ventral to it, a small nasal sac has developed. There are no nerve connections with the nasal sac.

No nerve knots were observed in this case.

The third, fourth, and fifth spinal ganglia are all deficient in the number of cells found when compared to the unoperated side. The third spinal nerve and the fourth are similar in behavior, growing out laterally and being deflected dorsally near the eye to ramify into peripheral tissue. The fifth nerve grows posteriorly, but it is questionable whether it reaches the limb or not. Its path could not be followed since many consecutive sections were torn at that point.

Its hypoplastic development suggests that it did not reach the limb. The sixth nerve, however, innervates the limb and, as would be expected, shows hyperplasia (see tables).

*Summary.*—The eye developed opposite the third and fourth segments. The optic nerve did not penetrate the retina but lies in a number of branches along the inner margin of the retina. No nerve knots were observed. The nerves normally forming the brachial plexus innervate neither the limb nor the transplant, and all corresponding ganglia show hypoplasia. The sixth nerve innervates the limb, and the sixth ganglion shows hyperplasia.

FIG. 16.—Photograph of section of LE 24, particularly showing the third spinal nerve as it deflected past the eye. *A*, cartilage around eye joined with muscle tissue. *S.N.*, spinal nerve.

## DISCUSSION

*Nerve knots.*—In every case but one, LE 24, one or more nerve masses developed. When these masses or knots are medial to the eye, they are generally penetrated by fibers from the sympathetic system at intervals along their entire length. These nerve knots are in all probability developed portions of the forebrain which happened to be transplanted with the eye. It might be suggested that the nerve knot is formed by the optic nerve itself as it grows out, and that its fibers are balled up into such a knot. One would be especially apt to come to such a conclusion after examining case LE 21, where the nerve knot, into which the optic nerve enters, is found between the layers of the retina. It is possible, however, that such anomalous development is due to some slight injury to the retina during the operation and that forebrain tissue came into more intimate contact with the nervous layer of the retina than would otherwise have been the case.

Further, the fact that in some cases (LE 44 especially) there are numerous nerve knots and but one of them connected with the optic nerve would support the contention that they are forebrain material and not optic nerve. The nerve knots not connected with optic nerves are similar in appearance and structure to those that are connected.

There is no apparent organization in these knots, and they seem to be passive in their behavior. As stated above, they may be connected with the sympathetic system; but in only one case (LE 44)

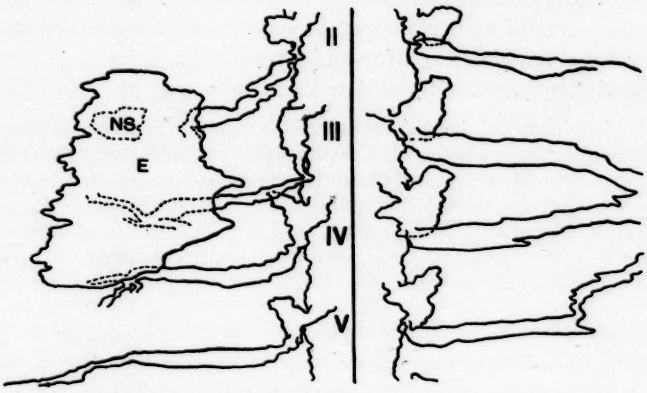


FIG. 17.—Reconstruction of LE 24. *E*, eye. *N.S.*, nasal sac

is there any connection of knots with spinal nerves, namely, where the second nerve penetrates the nasal placode after having passed through a knot, and where the fifth nerve enters another large knot, more caudal to the transplant. The connection between knot and nerve is rather intimate in the latter case, but in the former less so, since the nerve can be traced through the knot to the nasal sac.

*Optic nerve.*—In all cases but one, LE 24, an optic nerve was found to penetrate the retina in the normal manner and reach beyond the peripheral margin of the eye, from which it joins an adjacent nerve knot. It is significant to note that the exception in this case is the one in which no nerve knots were found. In LE 24 the optic nerve remains lying along the inner margin of the retina, not penetrating it at any point. These observations suggest the possi-

bility that forebrain material gives the necessary stimulus for the outgrowth of the optic nerve. Further operations have been performed in order to study this phenomenon in other specimens.

May and Detwiler (1925), using embryos in the tail-bud stage and placing eyes into the otic cavity, from which the otic vesicle had been removed, found the optic nerve to penetrate cranial nerve tissue. It is to be noted, however, that an eye in the otic cavity is placed in the immediate vicinity of outgrowing nerve tissue. Moreover, the eye is closer to the brain than when transplanted into the limb site. But before definite conclusions may be drawn concerning this point, more embryos must be examined. No descriptions of nerve knots are given by May and Detwiler.

Lewis (1907, pp. 261–62) describes the following:

> It often happened that the cut separating the optic vesicle from the brain was in such a plane as to include a small amount of brain tissue, the latter remaining attached to one side of the optic vesicle. The resulting differentiation of the two tissues, after transplantation, into eye and brain tissue, may go on without the formation of an optic stalk, the brain tissue remaining attached to the eye by its pigment layer. Or an optic stalk may differentiate with the brain tissue at one end and the eye at the other.

This description suggests a similarity to the cases in the present experiment, where the optic nerve connects the eye and nerve knot, which has been interpreted as being brain tissue.

May and Detwiler (1925) also ascribe a protuberance of the retina at the point of outgrowth of the optic nerve to an attractive influence of nerve centers. Case LE 1 shows that such distortion is possible even when there is no connection between the eye or optic nerve and nerve centers. It is very doubtful that the optic nerve is anything but passive in its reaction toward the central nervous system at the brachial level of the cord.

Uhlenhuth (1912) describes cases in which he placed eyes in the cervical region and in which he found the optic nerves growing out into long strands which sometimes join a spinal ganglion. He attributes the method of growth to the influence of both the transplant and substratum. His experiments, however, were performed on animals which had already become larvae, at which stage the optic nerve has already grown out, having been during its early develop-

ment subject to normal influences by its proximity to the forebrain.

The optic nerves in all observed specimens were much smaller than the normal. Since fibers from the ganglion cells contribute to the formation of the optic nerve, the decreased size must be due to the fact that there are fewer ganglion cells in the transplanted eyes.

*The eye as a whole.*—The eyes in all transplants developed the normal constituents. On the whole the transplanted eyes lacked the orderly arrangement of cells characteristic of the normal, case LE 24 showing the most disorderly disposition of all. The eyes seemed unaffected by the tissues of the host, which in some instances were allowed to come into contact with the peripheral border of the eye where the cartilage was lacking.

*The nasal placode.*—A nasal sac was formed in every case, though not always very fully developed. The greatest amount of development was shown by the nasal placode in LE 44, in which case it was innervated by a portion of the third spinal nerve. This is the only case in which the nasal sac received a nerve supply from the cord.

*The spinal ganglia.*—As a whole, the spinal ganglia show hypoplasia when deprived of the normal amount of peripheral stimulus, and hyperplasia when their nerves innervate an increased peripheral area. In a number of instances, however, the size of the ganglia differs unaccountably from what could be expected. Ganglion VI in LE 44 shows hypoplasia, though its normal peripheral stimulus does not seem to have been disturbed. Ganglion VIII in the same case shows hypoplasia, which is the opposite of what could be expected, since the nerve joins the brachial plexus. The nerve, however, is not small because the ventral root is large.

In LE 27 the second spinal ganglion shows a hypoplasia of nearly 50 per cent, for which there is no apparent reason. This is also true of the second spinal ganglion in LE 24.

The spinal ganglia in LE 21 show the most regular behavior of all the cases. The third spinal ganglion, which has lost its normal stimulus, shows hypoplasia; the fourth and fifth, which normally innervate the limb, are approximately normal in size; and the sixth, seventh, eighth, and ninth, whose peripheral stimulus has increased

because they innervate the limb, all show hyperplasia (see Tables I and II).

*The brachial plexus.*—In reviewing the cases described, it will be found that none of the nerves at the limb-level of the cord effects any connections with optic nerves. The brachial plexus exhibits the "preference" discussed by Detwiler (1922) in three of the cases where all or a part of the brachial plexus together with nerves from the lower levels of the cord innervates the limb.

Though the eye represented actively growing foreign tissue, it presented no stimulus to produce innervation by spinal nerves. In a number of instances nerves were described as approaching the eye and then being deflected. It might seem as though the eye exerted an actual repellent influence and caused the nerves to turn from it, but there is no proof that this deviation is the result of anything but mechanical opposition afforded by the eye in the direct path of the lateral growth of the nerve. However, if one concedes a "preference" in Detwiler's sense, it is possible to conceive of a lack of preference, or, what would be the same, repulsion. It is also possible that a tissue is more easily innervated by nerves situated nearest those which normally effect innervation and which therefore show "preference" than by nerves more remotely situated from the normal position.

In general, these results agree with those of Detwiler (1928), whose published results appeared as the present work was being completed. Detwiler found that when the right anterior limb bud of *Amblystoma* embryos is removed and an optic vesicle with the adjacent nasal placode is grafted three or four segments caudad to the normal limb site, the spinal nerves from higher limb levels grow caudally toward the limb site, in one case actually penetrating the nasal sac. Cases in which tail buds were transplanted four segments caudad to the limb site, the limb rudiments having been previously removed, showed evidence of a tendency for spinal nerves of slightly higher levels than the graft to grow slightly caudally; in other words, the direction of the nerves was changed, presumably through influence of the tail-bud tissues. Tail buds transplanted to the limb site failed to be innervated by spinal nerves.

In the present experiments, as already noted, the spinal nerves

in a number of instances seem to approach the eye only to be deflected, which weakens the idea of an attractive effect being produced by the eye tissues. The direction of the spinal nerves toward the eye may well be due to factors outside the eye, the eye actually exhibiting a repelling effect when the nerves come close enough. At any rate, marked evidence of an attraction between eye tissues and spinal nerves is lacking. We agree, however, with Detwiler's general conclusion that the factors or forces "concerned with the general direction of growth appear to be of a very general non-specific character, whereas those concerned with the actual connections with their end-organs appear to be much more specific in nature" (p. 38).

## SUMMARY

1. The optic vesicle with portions of the nasal placode was transplanted into the right limb site of *Amblystoma punctatum* in the limb-bud stage. The limb bud was transplanted a varying number of segments posterior to the normal position. Five specimens were sectioned and studied.

2. An eye and a nasal sac developed in the limb site in each case.

3. In all cases but one, LE 24, nerve knots, one or more in number, were found to have developed in connection with the transplant. The optic nerve, with the exception of LE 24, enters one of the nerve knots, and its fibers become inextricably interwoven with those of the knot. In LE 24 the optic nerve remained on the inner margin of the retina.

4. There are no demonstrable connections between optic nerves and spinal nerves.

5. In one case a spinal nerve penetrated the transplanted nasal sac.

6. The experiments tend to support Detwiler's view that there is a "preference" of nerves of the brachial plexus for the limb.

7. Spinal nerves with decreased peripheral sensory stimulus show ganglionic hypoplasia, while nerves with increased peripheral sensory stimulus show hyperplasia, as a general rule.

8. There is no evidence of specific attraction between the central nervous system and the transplanted eye or optic nerve.

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# THE RELATION BETWEEN EMBRYO VOLUME AND THE SUSCEPTIBILITY OF AMBLYSTOMA PUNCTATUM EMBRYOS TO POTASSIUM CYANIDE

(One figure)

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THE analysis of the effects of the cyanides on protoplasm has yielded valuable information concerning protoplasmic activity. This method of analysis becomes increasingly valuable as the nature of the action of these highly reactive substances on protoplasm becomes better understood. The literature on the biological effects of the cyanides is voluminous; in addition to extensive studies of a pharmacological nature, experimental work of considerable significance has employed cyanide in some form, as an agent in producing depression of oxidations, in connection with the problem of anesthesia, in form regulation, and in other physiological investigations.

Two principal effects of the cyanides have been emphasized: first, in suitable concentrations they are powerful inhibitors of oxidative metabolism, an action which was shown by Geppert (1889) to be the basis of their extreme toxicity for mammals; second, in concentrations which kill slowly, the processes of death are accompanied by visible changes, including disintegration of the tissues, in many of the simpler organisms, an effect which makes possible the detection of differences in susceptibility to their toxic action. Unfortunately some of the conclusions drawn from work with cyanides are weakened by the fact that no account is taken of the chemical structure of the inorganic cyanide in the solution employed. Whether or not the biological effects are due to the characteristics of the normal cyanide or of the isocyanide is, in some cases at least, important. Recent work (Emerson and Buchanan, 1927) indicates that the effect of solutions of potassium cyanide on biological oxidations more closely resembles the effect of an isocyanide than of the normal, cyanide. Moreover, attempts to interpret the visible and disintegra-

tive effects of the cyanides in terms of their depressive action on oxidations have raised serious objections, for the exact relationship between the depression of oxidations by cyanide and its disintegrative action is not clear.

It has been shown (Child, 1924) that in embryos of several forms and in the adults of the simpler organisms regional differences in susceptibility to the disintegrative action of cyanides correspond to regional differences in rate of oxidative metabolism. Loeb (1913) assumed this to be true, although he included other physiological conditions as being concerned in the relative susceptibility of cells. He states (pp. 92–93): "All the facts agree with the assumption that the egg is more sensitive to abnormal solutions when its rate of oxidations is high, or when certain developmental changes, e.g. cell divisions, take place than when it is at rest." There is also considerable evidence at hand which shows not only that disintegration by cyanide and other toxic agents is more rapid in regions in which the rate of oxidations is greatest but also that there is a distinct correlation between the rate of oxidations and the degree of depression of oxygen metabolism—the higher the rate of oxidations, the greater the depressive effect of the agent. However, certain lines of evidence suggest the possibility that the depressive action of the cyanides on oxidations and their killing and disintegrative action are relatively independent.

In former papers (Buchanan, 1926*a*) it was shown that, while ether and ethyl alcohol in some way oppose the depressive action of KNC on oxidations in *Planaria*, these two agents accelerate the disintegrative action of strong solutions of KNC. The inference is plain that, under the conditions of these experiments, the disintegrative effect of strong cyanide solutions is not a direct result of the action of KNC on oxidations. Still stronger evidence of the independence of the effect of the cyanides on oxidations and their visible killing action may be cited. Allen (1919), Hyman (1919), and Gray (1924) have shown that it is not possible to extinguish biological oxidations completely by means of cyanides. In solutions that are lethal within a few hours, the oxygen consumption of the organisms remains at a constant subnormal level. Furthermore, certain oxidative enzymes appear not to be affected by the presence of cyanide (Dixon and

Thurlow, 1925). Lyon (1904) demonstrated the existence of rhythms in susceptibility to cyanide in the sea-urchin egg, but Gray (1925) finds no corresponding rhythms in the rate of oxygen consumption. Lund (1921*b*) showed that in low oxygen concentrations the oxygen consumption of *Planaria* is depressed, while Child (1919) found that lack of oxygen accelerates the disintegration of *Planaria* in KNC.

The conflict between these two lines of evidence suggests that high susceptibility to the disintegrative action of KNC may depend on some protoplasmic condition or conditions which are in many cases coexistent with relatively high rate of oxygen metabolism. Specifically the problem may be stated: Do protoplasmic conditions or states other than high rate of oxidations make for increased susceptibility to the visible lethal and disintegrative action of KNC?

## MATERIALS AND METHODS

In the present paper are reported the results of a series of investigations on the effect of varying the osmotic pressure of the environment on the volume of the early embryos of *Amblystoma punctatum* and on their susceptibility to the lethal action of KNC. The experiments were carried on during the springs of 1926, 1927, and 1928; a preliminary account of the results appeared elsewhere. While still incomplete, the work has yielded results which appear to have an important bearing on the problem of cyanide susceptibility.

The early embryos of *Amblystoma punctatum* offer several advantages for this type of investigation. They are relatively large and spherical, or nearly so; swelling and shrinkage may therefore be readily measured with considerable accuracy. They are easily removed from the jelly capsules, leaving only the vitelline membrane as a protective covering. Penetration of this membrane and of the embryo may be judged by disintegration or the inhibition of development in the cyanide solution. Death changes can be detected early with the aid of a hand lens, and recovery following removal from the cyanide solutions is indicated by developmental changes. The disadvantages are: Eggs from different females, or collected at different times, vary considerably in their response to experimental conditions; this fact necessitates confining the number of embryos used in each experiment to the number deposited by a single female in a

single bunch, with consequent restriction in the number of solutions and concentrations that may be employed in each experiment. Furthermore, the embryos are relatively resistant to KNC, and strong solutions and relatively long exposures are required to bring about death and disintegration.

Two types of experiments were carried out: those dealing with the visible lethal effects of the cyanide in various solutions and those dealing with the effect of these solutions, or certain of them, on the volume of the embryos. The exact procedure followed in the first type of experiments was: For each experiment a large batch of embryos in an early stage (not later than the neural-plate stage) was selected and the embryos dissected free from the jelly, since preliminary work showed that the capsule affords some protection against KNC. The embryos were then allowed to stand several hours in cold, running tap water, during which time any that had been injured by the handling died. To freshly made solutions of amphibian Ringer's solution with buffer, cane sugar, and urea—all made up in both glass distilled and tap water—and to tap and distilled water was added sufficient KNC as dry salt make the desired molar concentration. No corrections were made, however, for the disappearance of the cyanide due to its combination with the sugar or other reagents in the solutions. The solutions were shaken thoroughly with air to insure equal distribution of the KNC and saturation with oxygen. The embryos previously prepared were then transferred to 250 cc. Stender dishes in tap water and the water replaced by 250 cc. of the prepared solutions. Depending on the number of embryos available, groups of ten, twenty, or twenty-five were placed in each dish, the number of different solutions used in each experiment being thus limited. All experiments were conducted at room temperature, and during the preparation of the solutions the water employed was brought to room temperature before being used. Hence, as regards temperature conditions, all lots of embryos within any given experiment were comparable. However, between different experiments the temperature varied with that of the room. Throughout the experiments the embryos were protected from direct sunlight.

After the solutions had been added to the dishes containing the embryos, the ground-glass lids were placed in position and sealed

with vaseline. Examination of the condition of the embryos was then made at suitable intervals, and a record made of all that showed indications of death or disintegration. Occasionally it was necessary to remove the lids of the dishes temporarily during the examinations.

In the experiments dealing with the effects of the various solutions on the volume of the embryos, the following procedure was followed: The embryos were dissected out of the jelly capsules as before. After standing for an hour or more in cold, running tap water, a certain number were selected at random and were transferred, by means of a wide pipette, to the bowl of a thistle tube mounted perpendicularly on a suitable support. The stem of the thistle tube had previously been plugged with a rubber stopper and filled with tap water. The embryos were allowed to settle in groups of two or three down the stem of the tube until they rested on the rubber stopper. Ordinarily twenty or twenty-five embryos were placed in the thistle tube at a time. These formed a column of embryos in the stem of the tube, which was then measured with a vernier caliper, jarring of the tube being avoided, the height of the column recorded, the thistle tube removed from the support, the water drained out of the bowl, and the bowl submerged in a 250 cc. Stender dish which had previously been filled with a desired solution. The embryos slowly rolled down the stem of the tube and entered directly from the stem into the solution. As soon as all had left the stem, the thistle tube was gently withdrawn, allowing the embryos to scatter in the Stender dish, and the thistle tube was washed out and returned to its support. The time of entry of the embryos from the stem of the tube into the solution was recorded. In the same manner other groups of embryos were handled and the height of the column formed in the stem of the thistle tube in tap water was measured, the embryos being placed in other types of solutions. After the lapse of a convenient interval of time the thistle tube was clamped in the support, the stem filled with the first solution referred to above, and the embryos transferred from the Stender dish to the thistle tube as before. The height of the column thus formed and the time were recorded, and the embryos were either returned to the solution or discarded, as the nature of the experiment required. The embryos in the other solutions were handled in the same way—the

duration of exposure to the solutions and the height of the column formed in the thistle tube in tap water and after exposure being the data recorded for each solution employed.

A brief consideration of this method is necessary. The principle is that of the haemocrit. Instead of centrifugal force, however, gravity is employed to separate the embryos from the solution. Since the same thistle tube was employed in each experiment, the height of the column of embryos before and after exposure to the solutions is an index of their combined volumes and of the changes in volume brought about by exposure to the solutions. The weight of the embryos compressed the lower members of the column somewhat; but since gravity was the force applied, this compression may be regarded as nearly identical in determinations of the height of the column formed by a given lot of embryos before and after exposure to the solutions and between different lots of embryos within the same experiment. The diameter of the bore of the stem of the particular thistle tube employed was 4.0 mm.

The accuracy of the method was tested as follows: Twenty embryos, gastrulae, were selected at random from a single bunch, and dissected out of the jelly capsules. The thistle tube was clamped in perpendicular with its stem filled with tap water. The embryos were then introduced in the manner described above, and the height of the column recorded. The tube was then removed, and its bowl submerged in a dish filled with tap water. After all the embryos had rolled out into the dish, the tube was again clamped in the support, and the operation of transferring the embryos from the dish to the stem repeated. Twenty consecutive measurements of the column formed by the same lot of embryos, the method in each case simulating the method employed in experiments involving exposure to the various solutions, yielded the following results: extremes, 34.0 mm. to 34.9 mm; standard deviation, 0.253 mm.; mean, 34.47 mm. plus or minus 0.38 mm. Differences between the first and later measurements of a single lot of embryos greater than 1.1 per cent are therefore to be regarded as significant.

It was found impracticable to remove the vitelline membrane in these experiments. The results in both types of experiments are therefore open to the objection that the entry of the substances may

be regulated by the permeability characteristics of the vitelline membrane. Whatever the influence of the vitelline membrane on the penetration of the substances, the fact remains that the embryos do die and do change volume inside the membrane and that the observations do reveal the influence of the substances on the embryo itself. In solutions in which the height of the column of embryos is increased, it was observed that the vitelline membrane was raised somewhat. However, when such embryos were placed in the thistle tube, inspection showed that the vitelline membranes were not turgid but that the embryos formed a compact column with each embryo in intimate contact with those adjacent.

## RESULTS

The various substances employed and the concentrations in the several experiments are shown in the accompanying tables, preliminary experiments having indicated that these concentrations were most useful for the purposes of the investigation. The distilled water used was distilled in glass and stored in clean glass containers. The tap water was drawn into glass containers and allowed to stand until in equilibrium with the air as regards temperature, oxygen, and carbon-dioxide content. As will be noted in the data, the results varied considerably in degree between different experiments of the same sort, but the order of effect of the various solutions was subject to little variation. The variations in degree are in part to be ascribed to differences between different groups of embryos which are not controllable, and in part to differences in handling and treatment after being brought to the laboratory.

As stated in a preliminary report of this work (Buchanan, 1926*b*), indications of death of the embryos differ among the solutions used. In 50 per cent Ringer's solution made up with KNC, and in all other solutions in which death progresses slowly, in blastulae death is first marked by the appearance of a gray cap at the animal pole. This cap spreads downward slowly and eventually involves the entire surface. A white spot soon appears at the animal pole. Sooner or later the embryo bursts, usually in the vegetal hemisphere, and the contents diffuse out as a fluffy gray cloud in the surrounding solution. The earlier the onset of death the sooner the bursting of the embryo, so the number of disintegrating embryos in a given lot is a

fair index of the relative effectiveness of the solution employed as compared with other solutions in the same experiment. In gastrulae and in embryos in which the neural plate is indicated the order of death is different. In these forms death begins at the blastopore, involving first the dorsal lip, from which region it spreads anteriorly

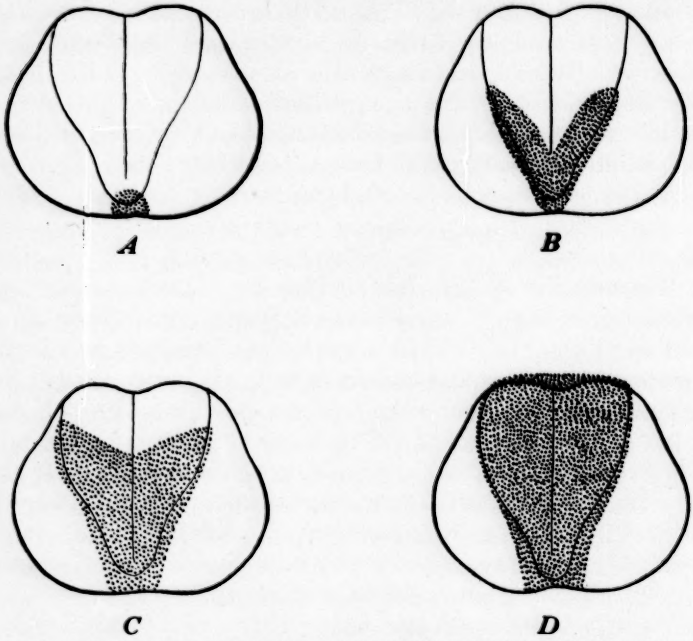


FIG. 1 *A–D*.—Four stages in the early lethal effect of KNC (M/250) made up in tap-water Ringer's solution diluted one-half. Redrawn from free-hand sketches. Stippled areas represent the regions visibly affected in the order given. Observations made during experiment No. 12.

and ventrad, eventually terminating in the disintegration of the embryo. In embryos in which the neural plate is distinct, the death changes occur first at the dorsal lip of the blastopore (Fig. 1*A*); from this region the external indications of death appear to spread forward, involving the neural plate, which may persist as a white area of dead cells for some time before other regions of the embryo are similarly affected. Figure 1, redrawn from free-hand sketches, indicates the usual early progress of death changes in such cases.

In full Ringer's solution and in 0.3 molar cane sugar solutions made up with KNC, death occurs only after extended exposure, 24 hours or more, usually much later. When death changes are finally brought about in such solutions, there is no clear evidence of differential susceptibility. The entire embryo slowly becomes a much lighter color, apparently almost simultaneously in all regions, and eventually bursts in the region of the yolk cells or the ventral body wall. The depressive action of the KNC is indicated by the fact that development is apparently almost totally inhibited very quickly when the embryos are placed in these solutions.

In solutions in which the embryos die very soon after being exposed, distinct differences in susceptibility cannot be readily detected. In such solutions the embryos usually burst within an hour, the contents diffusing out into the surrounding solution.

The relative effectiveness of KNC in Ringer's solution in two concentrations, made up in both tap and distilled water is shown in Table I. In Part A of the table are given fair samples of the nature of the results in individual experiments. All other tables given are compilations of data from such experiments. In Part B an attempt is made to show the relation between duration of exposure and the onset of disintegration. The data in the vertical columns are obtained in each case by dividing the time in minutes from the beginning of exposure to the first observation time which showed one or more embryos disintegrating, by the number visibly affected or disintegrating at that observation time. For instance, in the data for experiment No. 11, given in Part A, for distilled water+KNC, at the time of the first observation nine embryos had disintegrated. Since sixty divided by nine equals 6.6, this number serves as an index of the rapidity of appearance of visible death changes in this agent and is to be found recorded in Part B of Table I. Likewise, in Part A, for tap water+KNC, at the time of the first observation two embryos had disintegrated; hence the index figure for this solution in Part B, experiment No. 11, is thirty. Indices for the other solutions were arrived at in the same way. No claim of accuracy is made for this method; however, it serves a useful purpose in showing the relative rapidity of action of the various solutions within any given experiment.

Part C of Table I represents an attempt to show the relative susceptibility of the embryos to the various solutions employed. The data given in the table were obtained by adding the number of embryos visibly affected by death changes at each observation time

TABLE I A

TWO SAMPLE EXPERIMENTS ON THE DISINTEGRATIVE AND LETHAL ACTION OF VARIOUS SOLUTIONS ON *Amblystoma* EMBRYOS

(The figures represent the number of embryos visibly affected at each observation time)

EXPERIMENT NO. 11: BLASTULAE—M/250 KNC—20 EMBRYOS IN EACH

| | HOURS | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 5 | 10 | 18 | 24 |
| Dist. $H_2O$+KNC | 9 | 19 | 20 | 20 | 20 | 20 |
| Tap $H_2O$+KNC | 2 | 2 | 2 | 2 | 3 | 6 |
| Ringer-dist.+KNC | 0 | 1 | 1 | 2 | 5 | 14 |
| Ringer-tap+KNC | 0 | 0 | 0 | 0 | 1 | 7 |
| Ringer-dist. $H_2O$ | 0 | 0 | 0 | 0 | 0 | 0 |
| Ringer-tap $H_2O$ | 0 | 0 | 0 | 0 | 0 | 0 |
| Dist. $H_2O$ | 0 | 0 | 0 | 0 | 0 | 0 |
| Tap $H_2O$ | 0 | 0 | 0 | 0 | 0 | 0 |

EXPERIMENT NO. 18: NEURAL PLATE—M/125 KNC—25 EMBRYOS IN EACH

| | HOURS | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 12 |
| Dist. $H_2O$+KNC | 25 | 25 | 25 | 25 |
| Tap $H_2O$+KNC | 18 | 23 | 25 | 25 |
| Ringer-tap+KNC | 1 | 7 | 12 | 13 |
| ½ Ringer-tap+KNC | 7 | 11 | 18 | 25 |
| Ringer-tap $H_2O$ | 0 | 0 | 0 | 0 |
| ½ Ringer-tap $H_2O$ | 0 | 0 | 0 | 0 |
| Tap $H_2O$ | 0 | 0 | 0 | 0 |

for all observations covering the duration of the experiment, and dividing this sum by the number of observations. The figure thus obtained represents the average number disintegrated at each observation. Comparison of these data with those given in Part B shows that with very few exceptions the order of effectiveness of the various solutions is the same; i.e., solutions which cause embryos to disintegrate first also cause more to disintegrate during the course of an experiment.

## TABLE I B

INDEX OF DURATION OF EXPOSURE REQUIRED TO CAUSE DISINTEGRATION OF ONE EMBRYO*

| | No. of Experiment | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 20 | 19 | 18 | 12 | 7 | 8 | 6 | 11 | 17 | 31 | 16 | 33 |
| Stage of embryos | S.Y.P. | Bl. | N.Pl. | N.Pl. | Bl. | Y.P. | Bl. | Bl. | N.Pl. | S.Y.P. | Y.P. | Bl. |
| No. of embryos in each | 25 | 25 | 25 | 20 | 10 | 20 | 10 | 20 | 20 | 25 | 20 | 25 |
| Concentration of KNC | M/250 | M/125 | M/125 | M/250 | M/250 | M/250 | M/250 | M/250 | M/250 | M/250 | M/250 | M/250 |
| Dist. $H_2O$+KNC | 2.6 | 2.6 | 2.4 | 6.0 | 12 | 10.5 | 7.5 | 6.6 | 20 | 12 | ..... | 51.4 |
| Tap $H_2O$+KNC | 20 | 72 | 3.3 | 15 | 20 | 40 | 20 | 30 | 60 | 120 | ..... | — |
| ½ Ringer-dist.+KNC | 30 | 180 | ..... | 4.2 | 54 | 22.5 | 108 | ..... | ..... | — | ..... | ..... |
| ½ ringer-tap+KNC | ..... | ..... | 8.5 | 20 | 135 | 20 | 300 | ..... | 180 | ..... | 60 | ..... |
| Ringer-dist.+KNC | 120 | 120 | ..... | ..... | ..... | ..... | ..... | 120 | ..... | ..... | ..... | ..... |
| Ringer-tap+KNC | ..... | ..... | 60 | ..... | ..... | * | ..... | 1080 | — | ..... | — | ..... |
| Ringer–dist. $H_2O$ | 180 | — | ..... | ..... | ..... | ..... | ..... | — | ..... | — | ..... | ..... |
| Ringer–tap $H_2O$ | ..... | ..... | — | ..... | ..... | ..... | ..... | — | 60 | ..... | 960 | ..... |
| ½ Ringer–dist. $H_2O$ | — | — | ..... | — | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| ½ Ringer–tap $H_2O$ | ..... | ..... | — | — | ..... | ..... | ..... | ..... | ..... | ..... | 300 | ..... |
| Dist. $H_2O$ | — | — | — | — | — | — | — | — | — | — | ..... | — |
| Tap $H_2O$ | — | — | — | — | — | — | — | — | — | — | ..... | — |

* A dash (—) indicates that no embryos were visibly affected by the solution within the time limits of the experiment. S.Y.P. = small yolk plug; Bl. = blastulae; N.Pl. = neural plate; Y.P. = yolk plug.

TABLE I C

AVERAGE NUMBER OF EMBRYOS VISIBLY AFFECTED AT EACH OBSERVATION*

| | No. of Experiment | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 20 | 19 | 18 | 12 | 7 | 8 | 6 | 11 | 17 | 31 | 16 | 33 |
| Stage of embryos | S.Y.P. | Bl. | N.Pl. | N.Pl. | Bl. | Y.P. | Bl. | Bl. | N.Pl. | S.Y.P. | Y.P. | Bl. |
| No. of embryos in each solution | 25 | 25 | 25 | 20 | 10 | 20 | 10 | 20 | 20 | 25 | 20 | 25 |
| Concentration of KNC | M/250 | M/125 | M/125 | M/250 | M/250 | M/250 | M/250 | M/250 | M/250 | M/250 | M/250 | M/250 |
| Duration of experiment in hours | 8 | 7 | 12 | 10 | 11 | 14 | 9 | 18 | 10 | 2 | 16 | 12 |
| No. of observations | 5 | 5 | 4 | 7 | 6 | 6 | 6 | 5 | 7 | 2 | 6 | 2 |
| Dist. $H_2O$+KNC | 25 | 24.6 | 25 | 13.9 | 28.5 | 16.2 | 9.7 | 17.6 | 9.6 | 10 | ..... | 7 |
| Tap $H_2O$+KNC | 7.8 | 2.2 | 22.8 | 5.3 | 3.2 | 3.2 | 5.5 | 2.2 | 2.3 | 0.8 | ..... | 0 |
| ½ Ringer-dist.+KNC | 5.4 | 0.8 | ..... | 16.2 | 3.4 | 7.2 | 0 | ..... | ..... | ..... | ..... | ..... |
| ½ Ringer-tap+KNC | ..... | ..... | 15.3 | 4.2 | 2.0 | 6.7 | 1.4 | ..... | 0.8 | 0 | 1.2 | ..... |
| Ringer-dist.+KNC | 1.2 | 2.0 | ..... | ..... | ..... | ..... | ..... | 1.8 | ..... | ..... | ..... | ..... |
| Ringer-tap+KNC | ..... | ..... | 8.3 | ..... | ..... | ..... | ..... | 0.2 | 0 | ..... | 0 | ..... |
| Ringer–dist. $H_2O$ | 0.8 | 0 | ..... | ..... | ..... | ..... | ..... | 0 | ..... | 0 | ..... | ..... |
| Ringer–tap $H_2O$ | ..... | ..... | 0 | ..... | ..... | ..... | ..... | 0 | ..... | ..... | 0.2 | ..... |
| ½ Ringer–dist. $H_2O$ | 0 | 0 | ..... | 0 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| ½ Ringer–tap $H_2O$ | ..... | ..... | 0 | 0 | ..... | ..... | ..... | ..... | ..... | ..... | 0.4 | ..... |
| Dist. $H_2O$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ..... | 0 |
| Tap $H_2O$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* Ringer's solutions in both tap and distilled water with and without KNC. Tap and distilled water with and without KNC. S.Y.P. = small yolk plug; Bl. = blastulae; N.Pl. = neural plate; Y.P. = yolk plug.

Inspection of the data permits the following general conclusions: (1) Distilled-water solutions of KNC are more effective as disintegrative agents than are tap-water solutions. (2) Solutions of KNC in Ringer's solution, half-strength, made up in distilled water, are somewhat more effective as disintegrative agents than are such solutions employing tap water. (3) Ringer's solution, containing KNC, whether made up in tap or distilled water, is relatively ineffective, the least effective KNC solution being that made up in Ringer's in tap water. (4) Ringer's solution without the KNC does not kill the embryos within the time limits of these experiments.

A few experiments using double-strength Ringer's solution and also Ringer's diluted one-quarter and one-eighth were carried out. The results in the strong Ringer's are not clear because of the shrinkage of the embryos, with consequent wrinkling, and also because of apparent partial fixation. In the more dilute Ringer's the results were not appreciably different from those obtained with tap-water solutions of KNC.

Table II shows the relative effectiveness of KNC in solutions of cane sugar. This table was prepared from the data in all the experiments with cane sugar, and the figures in the vertical columns represent, as in Table I C, the average number of embryos disintegrating at each observation. Indirectly these data indicate the relative effectiveness of KNC in the presence of various concentrations of sugar. They show conclusively that sugar, like Ringer's solution, protects the embryos against the disintegrative action of the cyanide and that with increasing concentration of the sugar the protective action becomes greater. To regard this protective action of the sugar as solely due to its osmotic action is not justified, however, for undoubtedly cane sugar reacts with KNC to form an aldehyde, thereby removing some of the cyanide from the solution. However, the similarity between these results and those with isotonic Ringer's solutions suggest that the osmotic action is important in the protection afforded by the sugar.

For purposes of comparison between such osmotically active solutions as Ringer's and cane sugar and solutions isomolar with the sugar but developing materially less osmotic pressure, the disintegrative action of KNC made up in various concentrations of

urea was tested and compared with the action of KNC in sugar solutions isomolar with the urea and with Ringer's solution. The results are given in Table III, the data in which are derived in the same manner as those in Table I C, and Table II. The results show that the KNC-urea solutions are more effective disintegrating agents than

TABLE II

AVERAGE NUMBER OF EMBRYOS VISIBLY AFFECTED AT EACH OBSERVATION*

| | No. of Experiment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 15 | 16 | 41 | 12 | 7 | 8 | 6 | 14 |
| Stage of embryos | N.Pl. | N.Pl. | Bl. | N.Pl. | Bl. | Y.P. | N.Pl. | N.Pl. |
| No. of embryos in each solution | 20 | 20 | 20 | 20 | 10 | 20 | 10 | 20 |
| Concentration of KNC | M/125 | M/125 | M/250 | M/250 | M/250 | M/250 | M/250 | M/250 |
| Duration of experiment in hours | 11 | 21 | 18 | 10 | 11 | 14 | 9 | 9 |
| No. of observations | 6 | 4 | 5 | 7 | 6 | 6 | 6 | 7 |
| Dist. $H_2O$+KNC | ..... | ..... | 17.6 | 13.9 | 8.5 | 16.2 | 9.7 | ..... |
| Tap $H_2O$+KNC | 11.4 | 10.3 | 2.4 | 5.3 | 2.0 | 3.2 | 5.5 | 5.0 |
| M/o.375 sugar-tap+KNC | 11.4 | 9.3 | ..... | ..... | ..... | ..... | ..... | ..... |
| M/o.075 sugar-tap+KNC | 7.5 | 5.3 | ..... | ..... | ..... | ..... | ..... | ..... |
| M/o.15 sugar-tap+KNC | 3.0 | 0.8 | ..... | 0 | 0 | 0 | 0 | ..... |
| M/o.3 sugar-tap+KNC | 0 | 0 | 0 | ..... | ..... | ..... | ..... | ..... |
| M/o.3 sugar-dist.+KNC | ..... | ..... | 0.8 | ..... | ..... | ..... | ..... | ..... |
| M/o.15 sugar-dist.+KNC | ..... | ..... | ..... | 0 | 0 | 0 | 1.0 | ..... |
| M/o.6 sugar-tap+KNC | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 2.6 |
| M/o.6 sugar-tap $H_2O$ | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 0 |
| M/o.3 sugar-tap $H_2O$ | Separate tests; none disintegrated | | | | | | | |
| M/o.3 sugar–dist. $H_2O$ | Separate tests; none disintegrated | | | | | | | |
| M/o.15 sugar–tap $H_2O$ | ..... | ..... | 0.4 | 0 | ..... | ..... | ..... | ..... |
| M/o.15 sugar–dist. $H_2O$ | ..... | ..... | ..... | 0 | ..... | ..... | ..... | ..... |
| Tap $H_2O$ | ..... | ..... | ..... | 0 | 0 | 0 | 0 | ..... |
| Dist. $H_2O$ | ..... | ..... | ..... | 0 | 0 | 0 | 0 | ..... |

* Cane sugar solutions in both tap and distilled water with and without KNC. Tap and distilled water with and without KNC. N.Pl. = neural plate; Y.P. = yolk plug; Bl. = blastulae.

are the KNC-sugar or KNC-Ringer's solutions. In fact, the presence of urea in some way augments to some extent the disintegrative action of the solution. From these results it appears that urea and KNC are somewhat additive in their toxic effects.

From the foregoing data on the disintegrative action of KNC in various solutions, it appeared that a relation existed between the osmotic pressure of the solution and the action of the KNC. Since the effects of hypertonic and hypotonic solutions on the embryo are chiefly changes in its water content, and since hypertonic solutions

protect against, and hypotonic solutions accelerate, cyanide disintegration, a more rigorous investigation of the relation thus indicated was undertaken. Changes in volume of the embryos being an index of changes in water relations between embryo and solution, this second set of experiments was designed to test the effect on the

TABLE III

AVERAGE NUMBER OF EMBRYOS VISIBLY AFFECTED AT EACH OBSERVATION*

| | No. of Experiment | | | | |
|---|---|---|---|---|---|
| | 11 | 12 | 7 | 8 | 6 |
| Stage of embryos | Bl. | N.Pl. | Bl. | Y.P. | Bl. |
| No. of embryos in each solution | 20 | 20 | 10 | 20 | 10 |
| Concentration of KNC | M/250 | M/250 | M/250 | M/250 | M/250 |
| Duration of experiment in hours | 18 | 10 | 11 | 14 | 9 |
| No. of observations | 5 | 7 | 5 | 6 | 6 |
| Dist. $H_2O$+KNC | 17.6 | 13.9 | 8.5 | 16.2 | 9.7 |
| Tap $H_2O$+KNC | 2.2 | 5.3 | 3.2 | 3.2 | 5.5 |
| M/o.3 sugar-dist.+KNC | 0.8 | 0† | 0† | 0† | 1.0† |
| M/o.3 sugar-tap+KNC | 0 | 0‡ | 0‡ | 0‡ | 0‡ |
| M/o.3 urea-dist.+KNC | 18.8 | ..... | ..... | ..... | ..... |
| M/o.3 urea-tap+KNC | 12.2 | ..... | ..... | ..... | ..... |
| M/o.15 urea-dist.+KNC | ..... | 9.2 | 5.1 | 11.8 | 8.0 |
| M/o.15 urea-tap+KNC | ..... | 3.7 | 4.1 | 6.0 | 4.5 |
| M/o.075 urea-dist.+KNC | ..... | ..... | ..... | ..... | 0 |
| M/o.075 urea-tap+KNC | ..... | ..... | ..... | ..... | 0 |
| M/o.3 urea–dist. $H_2O$ | ..... | ..... | ..... | ..... | ..... |
| M/o.3 urea–tap $H_2O$ | 0.8 | ..... | ..... | ..... | ..... |
| M/o.15 urea–dist. $H_2O$ | ..... | 0 | ..... | ..... | ..... |
| M/o.15 urea–tap $H_2O$ | ..... | 0 | 0 | 0 | 0 |
| M/o.075 urea–tap $H_2O$ | ..... | ..... | ..... | ..... | 0 |
| Dist. $H_2O$ | 0 | ..... | 0 | 0 | 0 |
| Tap $H_2O$ | 0 | ..... | 0 | 0 | 0 |

* Urea solutions in both tap and distilled water with and without KNC; cane sugar solutions in both tap and distilled water with and without KNC; tap and distilled water with and without KNC. Bl. = blastulae; N.Pl. = neural plate; Y.P. = yolk plug.

† M/o.15 sugar-dist.+KNC.

‡ M/o.15 sugar-tap+KNC.

volume of the embryo of certain of the solutions employed in the first series of experiments. The method employed is described in detail above (p. 129). The results, in terms of percentage changes in height of the columns of embryos, of all experiments except certain preliminary tests of the method are compiled in Table IV.

It is perfectly clear from these data that KNC in the concentrations employed causes the *Amblystoma* embryos to swell. It is also clear that this swelling is approximately proportionally the same in hypertonic Ringer's solutions as in tap water, the latter being appar-

ently very nearly isotonic with the embryo. Furthermore, the swelling effect of the KNC and of distilled water are not additive. The changes in volume produced by the KNC are therefore not to be regarded as the consequences of changes in water relations due to the same causes as the changes effected by the osmotic pressure of the solution. In other words, the swelling of the embryos in KNC and their swelling in hypotonic solutions are different in nature;

TABLE IV

INCREASES AND DECREASES IN VOLUME OCCURRING IN *Amblystoma* EMBRYOS IN VARIOUS SOLUTIONS. (IN PER CENT)*

| | No. of Experiment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 20 | 30 | 31 | 33 | 17 | 18 | 19 | 16 |
| Stage of embryos | S.Y.P. | N.Pl. | S.Y.P. | Bl. | N.Pl. | N.Pl. | Bl. | S.Y.P. |
| No. of embryos in each solution | 20 | 20 | 25 | 25 | 25 | 25 | 25 | 20 |
| Concentration of KNC | M/250 | M/250 | M/250 | M/250 | M/250 | M/125 | M/125 | ..... |
| Duration of exposure | 30min. | 30min. | 1 hr. | 30min. | 3 hr. | 20min. | 20min. | 3 hr. |
| Dist. $H_2O$ | +30.5 | +4.7 | +27.2 | +20.0 | +41.0 | +27.5 | +25.5 | +30.8 |
| Dist. $H_2O$+KNC | +12.6 | +1.5 | +22.1 | +17.3 | + 9.4 | +19.1 | +17.1 | ..... |
| Tap $H_2O$ | + 1.0 | −0.9 | + 1.0 | 0 | + 8.1 | − 3.2 | + 1.2 | 0 |
| Tap $H_2O$+KNC | + 7.7 | +9.8 | + 8.7 | + 5.7 | +10.3 | + 7.8 | +11.6 | ..... |
| Ringer-dist. $H_2O$ | − 9.4 | −7.8 | −13.8 | ..... | −20.2 | −16.9 | − 8.3 | − 8.5 |
| Ringer-dist.+KNC | − 2.6 | −7.7 | −12.1 | ..... | ..... | − 5.4 | + 1.3 | ..... |
| ½ Ringer-dist. $H_2O$ | − 9.5 | ..... | ..... | ..... | −15.1 | − 9.3 | − 6.9 | − 7.8 |
| ½ Ringer-dist.+KNC | + 2.6 | ..... | ..... | ..... | ..... | + 5.4 | − 1.5 | ..... |
| ½ ether-tap $H_2O$ | ..... | ..... | − 1.0 | ..... | − 6.3 | + 0.6 | + 1.7 | ..... |
| ½ ether-tap+KNC | ..... | ..... | + 7.2 | ..... | + 1.5 | + 7.8 | + 6.0 | ..... |
| 2 Ringer-dist. $H_2O$ | ..... | ..... | ..... | ..... | −24.6 | ..... | ..... | −14.0 |

* S.Y.P. = small yolk plug; Bl. = blastulae; N.Pl. = neural plate.

furthermore, the exosmotic action of hypertonic solutions does not prevent the swelling effect of KNC. The resulting embryo volume is the algebraic sum of the two effects.

Blumenthal (1927) states that, in the case of unfertilized *Arbacia* eggs, "KNC . . . . caused a decrease in the volume of the eggs, when placed in hypotonic sea water, below that of the control exposed to sea water." His graphs, however, show (p. 318) that when the eggs were placed in hypotonic sea water after exposure to KNC in various concentrations they increased in volume distinctly more rapidly than did the controls. After 10 minutes the volume of eggs previously exposed to N/300 KNC had increased nearly 50 per cent, while the volume of controls had increased approximately 33 per cent. His data represent recovery phenomena and indicate that KNC

causes a decrease in egg volume. My own data show a directly opposite effect of KNC; in all cases except in distilled water, embryo volume was greater in the presence of KNC than in the same solution without the cyanide.

Comparison of the order of effect of the various solutions on embryo volume in Table IV with the order of effectiveness of these solutions in producing visible death changes shown in Tables I, II, and III shows that KNC has its greatest and most rapid disintegrative action in solutions which cause the embryos to swell, and its least effect in solutions which cause shrinkage. It appears, therefore, that the increase in volume produced by hypnotic solutions in some way accelerates the disintegrative action of the KNC. But the disintegration is not due merely to the bursting of the cells caused by excessive swelling, for distilled-water solutions of KNC are most effective in producing disintegration but less effective in increasing embryo volume than is distilled water alone. Experiments also show that the embryos tolerate distilled water for long periods of time.

Since solutions of KNC are distinctly alkaline, the suggestion arises that the effects noted here may be due to the effect of the alkalinity of the cyanide solutions. In testing this possibility it was regarded as undesirable to add buffers, or other reagents, to the various solutions in order to bring all to the same degree of acidity, because of the probability of thus introducing the specific effects of these reagents on disintegration and also because of thus altering the osmotic pressures with ensuing complications. The hydrogen-ion concentration of the solutions employed was determined in most experiments, particularly in the experiments on volume changes. The usual pH of these solutions, tested against standard solutions of chlorphenol red, bromthymol blue, and phenol red was:

| | pH |
|---|---|
| Distilled water | 6.6 |
| Distilled water plus KNC (M/250) | 8.6 |
| Tap water | 7.4 |
| Tap water plus KNC (M/250) | 8.6 |
| Ringer's solution (distilled water) | 7.8 |
| Ringer's solutions (distilled water) plus KNC (M/250) | 8.6 |
| One-half Ringer's (distilled water) | 7.8 |
| One-half Ringer's (distilled water) plus KNC (M/250) | 8.6 |
| One-quarter per cent ether in tap water | 7.6 |
| One-quarter per cent ether in tap water plus KNC (M/250) | 8.6 |

Examining the data on lethal effect and on embryo volume in the light of the pH of the solutions, it is seen that while the KNC solutions are of approximately the same pH, they vary greatly in their effectiveness as disintegrative agents, and also as regards their total effects on embryo volume. Any effect of hydrogen-ion concentration within the limits shown above is either excluded in the disintegration results or else masked by other properties of the solutions.

## DISCUSSION

That the lethal and disintegrative action of the cyanides is often accompanied by protoplasmic swelling has been repeatedly noted by Child and by others. Blumenthal (1927) regards the effect of HNC in causing swelling in the eggs of *Arbacia* as due to its effect on the cell boundary. Clear evidence is presented here that this swelling effect is not the primary cause of disintegration in KNC, for the change in volume of *Amblystoma* embryos due to KNC action occurs in hypertonic solutions in which disintegration occurs only after long-continued exposure.

A wide range of agents and conditions appear to augment the toxic action of the cyanides. Ether and ethyl alcohol accelerate the disintegration of *Planaria* in KNC solutions (Buchanan, 1926*a*), as does also lack of oxygen (Child, 1919). Although of doubtful significance in connection with the present problem, it may also be mentioned that Hunt (1907) found mice accustomed to alcohol-feeding much more susceptible to lethal doses of methyl cyanide than are controls, and Richards and Howland (Herter, 1907), report that KNC and indol are additive in producing death in dogs. Wilson (1925) holds that the disintegration of *Planaria* in KNC solutions is not due to the specific action of the KNC but to the hypotonicity of the solution for the internal tissues of the animal. Hypotonic solutions do not cause death and disintegration of *Amblystoma* embryos in the absence of KNC within the time limits obtaining in the experiments reported here; also, it may be pointed out that KNC, although its action is delayed, brings about disintegration in dilute Ringer's solution (one-half Ringer's), which causes some shrinkage of the embryos. Obviously Wilson's interpretation is inadequate to explain the action of the KNC on *Amblystoma* embryos,

for KNC causes disintegration in solutions that are somewhat hypertonic.

A considerable number of substances have been shown to be antagonistic to cyanide action. Mathews (1925, p. 44) points out that glucose, since it reacts with the cyanides, should tend to reduce their toxic effects. According to Krehan (1914), KNC and anesthetics are antagonistic in their effects on permeability. Loeb and Wasteneys (Loeb, 1913) found that KNC prolonged the life of sea-urchin eggs in cytolyzing hypertonic solutions of sodium and potassium chloride to which ammonium hydroxide had been added. The eggs had been previously stained with neutral red. Since the eggs were decolorized as quickly in the KNC solution as in the salt solution without the cyanide, Loeb concluded that the action of the cyanide in thus opposing the cytolytic action of hypertonic solutions is not due to any effect on the permeability of the egg. However, in the present work, where cyanide is the cytolizing agent and a hypertonic solution the protective agent, the antagonism cannot be so explained. The cyanide enters the embryo in the presence of hypertonic solutions, for development is profoundly inhibited and the embryonic volume is affected by the cyanide to approximately the same extent as when the KNC is applied in tap water. Hypertonic solutions must, therefore, be included in the list of conditions that are antagonistic to cyanide disintegration, but the antagonism is not directly related to opposite effects on permeability.

An examination of the experimental evidence presented here leads to the conclusion that in some way susceptibility to KNC in *Amblystoma* embryos is intimately related to the relative water content; the higher their water content the greater the susceptibility. The experimental facts which support this conclusion are: hypertonic solutions which cause decreases in volume protect the embryos against the killing action of KNC; hypotonic solutions in which increases in volume occur accelerate the disintegrative action of KNC; solutions of urea isomolar with protective sugar solutions show considerably less protective action against KNC; solutions of ether which do not materially affect the volume of the embryos do not accelerate cyanide action until after prolonged exposure. The possibility that the differences in susceptibility to KNC in these

solutions may be due to differences in their effects on the oxidative rate of the embryos is not totally excluded but is exceedingly improbable. If rate of development is regarded as an index of metabolic rate, then hypertonic and hypotonic solutions in the absence of KNC have no appreciable effect when acting during the comparatively short periods employed in these experiments. Another possibility may be considered, namely, that in the case of hypertonic solutions the protective action may be due to a possible failure of KNC to depress oxidations in the presence of hypertonic solutions. No experimental evidence concerning this possibility has been sought. It may be pointed out, however, that even if such a relation is subsequently shown to exist, it would be difficult to reconcile it with differential susceptibility in the several solutions employed without reference to the differences in water relations in these solutions.

As to the mechanism involved in cyanide action whereby increased water content of the embryo makes for increased susceptibility and decreased water content tends to prevent its lethal action there is no evidence at hand. It was shown by Heilbrunn (1920) that protoplasmic viscosity in sea-urchin eggs is increased by hypertonic solutions and that KNC also causes an increase in viscosity. While there is no evidence at hand concerning the effects of these two agents on protoplasmic viscosity in the *Amblystoma* egg or embryo, it is obvious that in the absence of such evidence, the results presented here cannot be interpreted in the light of the effect of these two agents on viscosity of sea-urchin eggs.

If the conclusion that the effectiveness of KNC as a lethal and disintegrative agent is intimately related to the water content of the *Amblystoma* embryo is correct, the differential susceptibility of different regions of the embryo in solutions which kill slowly, as shown in Figure 1, appears to indicate that those regions which are first affected have a relatively higher water content than regions affected later. In other words, the indication is that the osmotic pressure, or at least the water content, of highly susceptible regions is greater than that of regions less susceptible. So far as the writer is aware, there are no data available on the relative water content or relative osmotic pressure along the long axis of embryos or of adult forms in which gradients in susceptibility to KNC have been demon-

strated.[1] Apico-basal differences in sap concentration, however, have been demonstrated in plants; e.g., some work of Fernald (1925) indicates that the sap concentration is greater in apical sprouts of tubers and privet than in more basal sprouts. However, the fact is well known that in protoplasm in general, relatively high water content, particularly as regards adsorbed water, is usually associated with relatively high metabolic activity, a phenomenon which is by some investigators attributed to hydrogen-ion effects on biocolloids. Hence, even if it be true, as these data indicate, that relatively high susceptibility of *Amblystoma* embryos to KNC disintegration indicates only relatively high water content, differential susceptibility, shown in Figure 1 for example, serves as a useful but indirect index of physiologic activity.

For the present this interpretation of susceptibility based on relative water content is confined to the susceptibility of *Amblystoma* embryos to lethal solutions of KNC, although the statement by Child (1915, p. 73), that *Planaria* are more susceptible to KNC in distilled water, and less susceptible to KNC in 5 per cent sea water, than in well water, and the recent report of Wilson (1925) suggest the possibility that water relations may also be concerned in the differential susceptibility of *Planaria* to KNC. Susceptibility to lack of oxygen, to anesthetics, to ultra-violet light, and other toxic agents, may be expected to involve other and specific effects, and may be the result of quite different modes of action. However, the probable relation between water content and KNC susceptibility which is indicated in the present work offers a suggestion that may be useful in interpreting occasional cases of lack of parallelism between relative rate of oxidative metabolism and relative susceptibility to KNC, e.g., the susceptibility results of Lyon (1904) when compared with the oxygen consumption results of Gray (1925), discussed above (p. 127). The suggestion is offered that this absence of parallelism

[1] Hatai (1924) reports that in a Japanese earthworm the water content is least in the anterior region, increasing in the mid-region, and decreasing again toward the posterior end. Hyman (1916) found in certain oligochaetes that the anterior and posterior ends are more susceptible to KNC disintegration than the mid-region. Hatai suggests a possible association between low water content and high susceptibility. Obviously, my results recorded here point to an interpretation which is the reverse of Hatai's suggestion.

may be due to a correlation of low value between rapidity of oxidations and water relations during cleavage.

## CONCLUSIONS

Data on the lethal and disintegrative effect of KNC solutions made up in tap and distilled water, and in tap and distilled water solutions of cane sugar, Ringer's, urea, ether, and NaOH, on the early embryos of *Amblystoma punctatum* are given. The effect of these solutions on the volume of the embryos was also tested by measuring changes in height of a column of the embryos brought about by exposure to the solutions. The facts show that death and disintegration are more rapid in solutions in which embryo volume is increased, and less rapid in solutions in which embryo volume is decreased. In other words, hypotonic solutions accelerate, and hypertonic solutions protect, the embryos against the lethal and disintegrative action of KNC.

These facts indicate that the acceleration or protection thus afforded are in some way associated with the relative water content of the embryos. The inference is, therefore, that differential susceptibility to KNC disintegration is indicative of a differential in water content of the affected cells.

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